MICRORNAS: INNATE IMMUNE MODULATED RESTRICTION FACTORS OF SIV INFECTION

by
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Abstract

Macrophages play a major role in the innate immune response to infection. CD4+ T cells and macrophages are the target cells for HIV, the virus that causes AIDS, and cause establishment of infection in the brain. HIV infection of the brain often leads to chronic inflammation and HIV-associated neurocognitive disorders (HAND). Our lab has developed an SIV macaque model of HIV to study CNS disease and the role of macrophages and other cell types involved in these disorders.

Host cellular restriction factors inhibit HIV replication, and miRNAs are a member of this group. These small RNA molecules fine-tune gene expression through targeting 3' UTRs of mRNAs and inhibiting translation and/or causing mRNA degradation. Recently, there has been increased interest in miRNA regulation of viruses due to their role in pathogenesis of HIV-1 and other viruses.

In our studies, microarray analysis demonstrated levels of specific miRNAs that differ between monocytes (HIV-restricted) and macrophages (HIV-permissive) immune cells. These results suggest miRNAs may restrict virus infection, and also limit infection within target cell populations. Several miRNAs affect IFNβ, a cytokine induced early after HIV infection. Many genes are activated by IFNβ, and its regulation is critical for controlling immune signaling. miRNAs, miR-26a, -34a, -145 and –let7b have predicted binding sites in the IFNβ 3' UTR. An ELISA showed each of these miRNAs regulates IFNβ protein levels and RT-qPCR data demonstrates that mature miRNAs miR-26a, -34a and –let7b

were induced by IFNβ, indicating that IFNβ signaling induces miRNAs that then regulate expression of the protein.

We also identified miRNAs that target SIV RNAs and inhibit virus replication. The SIV 3' UTR contains functional binding sites for four different miRNAs, miR-29a, -29b, -9 and -146a, determined by RNA immunoprecipitation. Using primary macrophages, we found each miRNA reduced intracellular viral RNAs, as well as production of virus in supernatants of infected cells. RT-qPCR assays indicated modulation of mature miRNAs and primary miRNA transcripts by IFNβ and TNFα. These results demonstrate transcriptional regulation of miR-29a, -29b, -9 and -146a, four miRNAs that regulate virus replication in cells essential for immune signaling.

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I. INTRODUCTION

microRNAs

microRNAs (miRNAs) are ~22-nucleotide non-coding RNA molecules found in plants and animals that fine-tune expression of proteins within a cell. miRNAs regulate protein expression canonically through binding to the ends (3' untranslated regions/UTRs) of target mRNAs and decrease protein production by either mRNA degradation or translation suppression [1]. miRNAs were reported in 1993 in describing gene regulation in a developmental study of *Caenorhabditis elegans* (*C. elegans*) [2], a model organism used commonly used for genetic analyses. The majority of these small RNAs are initially transcribed by RNA Polymerase II which produces long primary miRNAs (pri-miRNAs) [3]. The mature miRNA sequence within the pri-miRNA forms a hairpin structure and while in the nucleus, the Drosha-DGCR8 protein complex [4] recognizes the hairpin. Drosha, with its RNase III activity, cleaves the pri-miRNA into an ~80-nucleotide precursor miRNA (pre-miRNA), removing the hairpin from the nascent transcript. The pre-miRNA is then transported from the nucleus to the cytoplasm by Exportin 5 [5], a nucleocytoplasmic transport protein. In the cytoplasm, the pre-miRNA hairpin is further processed by Dicer [6], another enzyme with RNase III activity, which removes the loop joining the two arms of the pre-miRNA duplex. One strand of the duplex is loaded into the RNA-induced Silencing Complex (RISC). RISC is a complex of several proteins that uses the miRNA strand to locate and bind to mRNA target(s). The first 2-8 nucleotides at the 5' end of the miRNA contains the seed region. The overall complementarity of the seed region with the seed-binding region in a target mRNA sequence is the most important factor in determining binding specificity [7]. There are

estimated to be over 1000 human microRNAs [8] predicted to target approximately 60% of the genome [9, 10]. miRNAs are present in all human cell types and are involved in processes ranging from organism development [11, 12] to antiviral defenses [13-17] and viral pathogenesis [18-21].

microRNAs related to viral infection and pathogenesis

miRNAs have been shown to regulate infection by many viruses including influenza, herpes viruses, Hepatitis C (HCV) and HIV-1, by reducing the replication of the virus and limiting the spread of infection to uninfected cells. miRNAs miR-323, -491 and -654 inhibit replication of H1N1 influenza A viruses through binding to a conserved region of the PB1 gene [20]. The protein encoded by this gene is an essential subunit of the viral RNA Polymerase I that is required for transcription of the viral genome. In addition, infection of human lung cell lines with H1N1 influenza A virus affects expression levels of many miRNAs [22], and mice infected with different strains of influenza A also show changes in miRNA expression [23]. A Kaposi's sarcoma-associated herpesvirus (KSHV), and Epstein-Barr virus (EBV), contain genes regulated by miR-1293 [24] and miRNAs in the miR-17 family [25], respectively. HCV presented an interesting case where miR-122 binds the 5' UTR of HCV and increases virus replication [18]. An antagonist of this miRNA has been through clinical trials and will be available to treat HCV early in 2014. HIV-infected individuals show changes in miRNA profiles compared to uninfected individuals [26, 27] and specific miRNAs have been shown to target HIV-1 mRNA sequences and reduce virus production [19, 28-31].

History of HIV-1

Human Immunodeficiency Virus (HIV) is the virus that causes Acquired Immunodeficiency Syndrome (AIDS). Simian Immunodeficiency Virus (SIVcpz) is a closely related lentivirus that infects chimpanzees in Africa. Phylogenetic studies of SIVcpz and HIV-1 strains suggest there have been four cross-over events of SIV into humans [32] most likely caused by hunting of infected non-human primates [33]. Because of the genetic relationship established between HIV-1 and SIV, it is now understood that all human HIV-1 infections were acquired from one particular chimpanzee subspecies and that chimpanzees are the natural reservoir for SIVcpz, the virus from which HIV-1 originated [32, 34].

Based on genetic evaluation of different virus strains, HIV-1 is estimated to have appeared in humans in the 1920s [34] and HIV/AIDS was first recognized in patients in the United States in 1981. The World Health Organization 2011 report shows there are approximately 34 million people currently infected with HIV. The region of the world most impacted by HIV has been sub-Saharan Africa, but this disease became, and continues to be, a global pandemic. Since the peak of the pandemic in 1997, the total global number of new HIV infections has been declining [35]. A decline in new infections beginning in 2005-2006 has continued along with decreasing AIDS-related deaths. While these numbers are declining, there were still 2.5 million new infections in 2012 and over 1.5 million deaths [35], demonstrating the continuing impact of this virus on the world population. The decline in AIDS-related deaths is in large part due to the development of Highly Active Antiretroviral Therapy (HAART) in 1996, which is a cocktail of three or more drugs that target different stages of the viral life cycle. HAART

transformed HIV/AIDS from a deadly disease to one that is managed for decades as a chronic disease. Due to HAART the number of AIDS-related deaths is declining, but the prevalence of people living with HIV/AIDS is increasing. Despite the effectiveness of current therapies in controlling virus replication and prolonging life, HIV-associated neurocognitive disorders (HAND) are still prevalent. Although fewer patients suffer from HIV-associated dementia (HAD), the most serious of CNS disorders, a greater number of patients are being diagnosed with other milder neurological disorders that affect the ability to work and overall quality of life.

HIV Infection Cycle

HIV was identified as a member of the Lentivirus genus of viruses [36] named for their characteristic long incubation periods prior to manifestation of disease. The first few weeks after infection with HIV is called the acute phase. During this time, individuals normally present with influenza-like symptoms and have high levels of circulating virus. These symptoms are accompanied by a rapid and dramatic decline in the number of CD4+ T cells. Innate and adaptive immune responses impact infection and the acute phase of infection is followed by an asymptomatic period during which there is an increase in CD4+ T cells and lower levels of virus found in the blood. The asymptomatic phase can last from months to years. The late stage of disease is AIDS, characterized by a dramatic rebound in viral load and a severe decline in number of CD4+ T cells. Individuals often succumb to infection by opportunistic pathogens such as bacteria and fungi that a person with a healthy immune system could successfully control.

HIV infects cells of the immune system including CD4+ T cells and cells of the myeloid lineage such as monocytes, macrophages and dendritic cells. In the peripheral blood and in lymph nodes CD4+ T cells are the major infected cell type. The loss of a large population of these immune cells leaves the individual vulnerable to multiple opportunistic infections and uncontrolled cancer cell growth [37]. In contrast to the periphery, monocytes and microglia/macrophages are the major infected cell type in the central nervous system (CNS) and these cells are involved in the inflammatory response to infection [38]. Infected, activated monocytes are able to cross the blood-brain barrier and enter the brain where they mature into perivascular macrophages. These cells support productive infection and spread virus to resident macrophages (microglia) and astrocytes [38]. Infected cells as well as bystander cells produce inflammatory cytokines, such as interferon beta (IFNβ), the main type I IFN produced in the brain [39]. The role of monocytes and macrophages during infection is important to understand, as these cells may contribute significantly to the clinical and pathological disease in the CNS of many patients with AIDS stage of disease as well as a large number of HIV-infected individuals on HAART who have HIV associated cognitive disorders (HAND).

HIV Restriction factors

During acute infection by various types of pathogens, the innate immune response fights the invading organism by stimulating the production of antiviral factors, such as IFNα/β. This triggers a cascade of factors to aid in clearing the infection. In addition to the interferon response, there are host cell restriction factors that also limit HIV/SIV infection. Restriction factors are proteins made by the infected cell that are able to target

the virus at specific stages of its life cycle and prevent virus spread to naïve cells. There are currently four known restriction factors of HIV infection: 1) APOBEC3G incorporates into nascent virions where it inhibits integration of the viral genome and/or production of functional viral proteins. The mechanism of action is through deamination of cytidines in the viral genome, resulting in guanidine to adenine hypermutations [40]; 2) TRIM5a interacts with the viral capsid protein and disrupts uncoating of the capsid [40]. This protein is only active against SIV, as the human analogue contains a mutation rendering the protein inactive [40]. 3) Bst-2/Tetherin is the third host restriction factor and blocks release of virions from cells by preventing physical detachment from the plasma membrane [40]. 4) The most recently discovered host restriction factor to HIV infection is SAMHD1. To date, the body of research on this protein suggests that SAMHD1 interferes with HIV reverse transcription by depleting the host cell dNTP pool [40]. microRNAs (miRNAs) are also host restriction factors of HIV. There has been increased interest recently in the effect of miRNAs on HIV infection and pathogenesis. It has been demonstrated that there is differential expression of miRNAs in resting vs. activated CD4+ T cells [41, 42] as well as in monocytes and macrophages [43, 44]. Some or all of these miRNAs affect cellular permissiveness to HIV-1 infection, and have been predicted and/or demonstrated to directly target HIV-1 mRNAs and specific miRNAs that reduce production of HIV-1 [19, 28-31, 45, 46].

Using varied in vitro assays we identified miRNAs with functional binding sites in the SIV genome. Presumably through targeting of SIV mRNAs, the miRNAs cause degradation of the SIV transcripts. Because all SIV mRNAs contain an identical 3' UTR, all transcripts are targets for the miRNAs. Reducing the numbers of various transcripts

results in an inhibition of virus replication. This work also demonstrates that some of these miRNAs regulate cytokine expression, and each miRNA analyzed here is induced by innate immune signaling.

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II. MicroRNA Regulation of IFNβ Protein Expression: Rapid and Sensitive Modulation of the Innate Immune Response

MicroRNA Regulation of IFNβ Protein Expression: Rapid and Sensitive Modulation of the Innate Immune Response

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[3]Abbreviations used in this paper: ARE (adenylate-uridylate-rich element), miRNA (microRNA), MRE (microRNA recognition element), and poly I:C (polyinosinic:polycytidylic acid)

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Abstract

Interferon beta (IFNβ) production is an inaugural event in the innate immune response to viral infections, with relatively small fold changes in IFNβ expression resulting in the activation of important antiviral signaling cascades. In our rapid SIV/macaque model of HIV encephalitis, virus enters the CNS within four days of infection, accompanied by a marked IFNβ response that wanes as SIV replication is controlled. The centrality of IFNβ to the innate antiviral response in the CNS combines with the potential inflammatory damage associated with long-term activation of this pathway to suggest that IFNβ may be subject to regulatory fine-tuning in addition to well-established transcriptional and message stability mechanisms of regulation. Here, we present for the first time evidence that microRNAs, including miRs -26a, -34a, -145, and let-7b, may directly regulate IFNβ in human and macaque cells. In primary primate macrophages, the main cell type implicated in HIV and SIV infection in the CNS, specific miRNAs reduce, while miRNA inhibitors enhance, IFNβ protein production. The potential biological significance of this regulation is supported by evidence of an apparent negative feedback loop, with increased expression of three IFNβ-regulating miRNAs by primate macrophages exposed to recombinant IFNβ or stimulated to produce IFNβ. Thus, miRNAs may contribute significantly to the regulation of IFNβ in innate immune responses.

Introduction

Acute retroviral infection provokes rapid and striking innate immune responses in what has been termed a "cytokine storm" [1]. These responses are crucial in determining the course of disease, as a delicate balance must be achieved between pro- and anti-inflammatory processes. The former must be sufficiently ferocious to dampen viral replication and impede further infection, while the latter are needed to prevent the tissue damage inherent in chronic activation of the immune system. In our rapid SIV/macaque model of HIV encephalitis, we have shown that both virus and the innate response are present in the central nervous system early in acute infection [2-4]. In addition, HIV and SIV infection of macrophages induces IFNβ, which in turn evokes downstream antiviral responses [2-8].

The cytokine IFNβ, with both pro- and anti-inflammatory roles, is the main Type I interferon induced during the initial innate response to retroviral infection of the central nervous system [9]. IFNβ mRNA and protein levels rise two- and four-fold, respectively, in response to viral replication during the acute phase of infection, followed by a decline during the asymptomatic phase [2, 4]. However, these relatively small fold-changes produce multiplicative effects on downstream effectors such as the antiviral protein MxA. IFNβ is also crucial for induction of anti-bacterial defenses [10, 11].

The pivotal nature of IFNβ and the magnified effects of its differential regulation suggest that intricate modulatory mechanisms have evolved to regulate its production. Indeed, over several decades, studies have elucidated numerous transcriptional and post-transcriptional strategies for IFNβ regulation [12]. Maniatis and Whittemore showed that an adenylate-uridylate-rich element $(ARE)^3$ in the 3'UTR of the IFNβ mRNA was partly

responsible for message degradation [13]. The IFNβ ARE may be the binding site for destabilizing ARE binding proteins (AUBPs) such as tristetraprolin [14]. Interestingly, the early work on these *cis*-acting elements in the 3'UTR also presaged an additional regulatory possibility: translational modulation by microRNAs (miRNAs)[3].

miRNAs are small RNA regulatory molecules, on average 22 nucleotides in length, that, when integrated into protein complexes known as RNA-induced silencing complexes (RISCs), bind to partially complementary sequences in the 3' untranslated regions of target mRNAs and thereby contribute to gene regulation by inhibiting translation and destabilizing transcripts [15, 16]. The human genome may encode over one thousand potentially functional miRNAs [17], and each mature miRNA may have the ability to regulate the expression of many genes. An estimated one-half of all protein-coding transcripts are thought to be subject to miRNA regulation [15]. Among several features predicting miRNA regulation of a given 3'UTR is the presence of an AU-rich sequence [18] such as that found in the transcripts of IFNβ and many other cytokines [19]. AU-rich elements are relatively unstructured and may thereby enhance miRNA target site accessibility for miRNA-containing ribonucleoproteins. Recent work has identified several potential cytokine miRNA targets [19], with experimental confirmation reported for IL-10 [20] and the p35 subunit of IL-12 [21]. Indirect effects of miRNAs on cytokines have also been reported [22, 23]. IFNβ is intimately involved in miRNA regulation, modulating the expression of numerous miRNAs [24] as well as the miRNA processing enzyme Dicer [25]. To date, no observations of direct effects of miRNAs on IFNβ have been published, although indirect effects of miR-146a on the Type I interferons have been reported [26, 27].

The importance of IFNβ regulation in the innate immune response to HIV and SIV and the presence of an extensive adenylate-uridylate-rich region in the IFNβ 3'UTR prompted us to investigate the role of miRNA in direct regulation of IFNβ. We used miRNA target prediction algorithms to identify several miRNAs with potential recognition sites in the IFNβ 3'UTR, and we screened for miRNAs expressed and differentially regulated in primary macrophages following exposure to IFNβ. Of these miRNAs, four silenced through the 3'UTR in a reporter assay and affected secretion of IFNβ protein by primary macrophages. Treatment of primary macrophages with IFNβ upregulates the expression of miR-26a, -34a, and let-7b, suggesting a negative feedback loop for the regulation of IFNβ protein. Treatment with polyinosinic:polycytidylic acid (poly I:C), a dsRNA stimulator of innate immune responses including Type I interferons, similarly resulted in miRNA upregulation, and in a time frame consistent with IFNβ-mediated regulation. These findings may have significant implications for the fine-tuning of the innate immune response during retroviral infection and, potentially, for the therapeutic modulation of innate immune responses.

Results

Predicted microRNA recognition elements in the IFNβ 3'UTR

To assess the potential for miRNA regulation of IFNβ, we sought miRNAs that could interact with miRNA recognition elements (MREs)[3] in the IFNβ 3'UTR. We used multiple prediction algorithms, including miRanda [28], RNAhybrid [29], and microInspector [30], to search for target sites in the macaque and human IFNβ 3'UTRs. We also queried PITA [31], which incorporates 3'UTR folding parameters into its predictions. The different target prediction algorithms place varying emphases on seed sequence complementarity, heteroduplex free energy of binding, location and size of internal loops and bulges, and accessibility of the target site (as predicted by RNA folding).

More than 200 total miRNA-mRNA target duplexes were predicted by these algorithms. Most target sites were concentrated in two clusters in the 5' half of the IFNβ 3'UTR (Figure 1, boxes). To pare this list of predictions to an experimentally tractable size, we next considered only those predictions shared by three prediction programs, and we further reduced the number of candidates by selecting those likely to be expressed in macrophages, a cell type of central importance to the innate immune response, HIV/SIV CNS disease, and IFNβ production [32]. The likelihood of expression in macrophages was assessed according to reported miRNA expression in CD14+ cells [33] and our own preliminary results from miRNA microarrays with both human and macaque macrophages (K. W. Witwer, unpublished data). miRs -26a, -34a, -145, -181a, -198, and let-7b were selected for initial study.

As is the case for most of the other predicted miRNAs, the MREs for macrophage-expressed miRNAs later shown to target the UTR (see below) are located in the target-rich 5' half of the IFNβ 3'UTR (Figure 1). Importantly, only two nucleotide differences distinguish the human and macaque 3'UTRs in this region (positions indicated by asterisks in Figure 1). Neither of these nucleotides is predicted to bind the 5' seed (6-8 nucleotides) of the targeting miRNA, although a G-A (macaque-human) difference in the miR-34a binding site changes a "wobble" G-U base pairing outside the seed region to a Watson-Crick interaction, potentially lending a marginally more favorable binding energy to the human miR-34a:target pair. This near-identity of the targeted sequences in human and macaque, combined with the high level of miRNA conservation in primates, suggests that observations made in one species translate well to the other.

miRNA mimics silence reporters containing IFNβ 3'UTR sequences

To examine the functional effects of these miRNAs, we used reporter assays. The macaque IFNβ 3'UTR sequence was cloned 3' to a luciferase reporter gene in a dual luciferase expression vector and transfected into HEK-293T cells. Four of the candidate miRNAs (miRs -26a, -34a, -145, and let-7b), when added exogenously as miRNA mimics, reduced the expression of luciferase in HEK-293T cells (Figure 2a) compared with no-miRNA controls and a control *Caenorhabditis elegans* miRNA with nucleotide composition similar to that of the mimics of interest. At 10nM transfected mimic, let-7b and an equimolar mixture of the four miRNAs significantly reduced luciferase expression ($p<0.001$). At 40nM, all four miRNAs reduced expression significantly at $p=0.01$ or

lower. miR-34a and the equimolar mixture achieved significant dose-dependent reduction in luciferase ($p<0.01$ and $p<0.05$, respectively). The apparent lack of dose dependence for other miRNAs may indicate that the lower concentration was sufficient to saturate binding sites and achieve maximal effect. The equimolar mixture (10nM = 2.5nm each, 40nM = 10nM each) effected a greater control than any single mimic, suggesting a cooperative effect of multiple microRNA recognition element occupancy, as has been reported previously [34]. Predicted binding sites for let-7b, miR-26a, and miR-145 overlap in a region near the 5' end of the UTR, while the miR-34a MRE is located in a separate region, just 5' to the AU-rich region.

To confirm the specific interaction of native miRNAs with the predicted MREs, we designed defective MREs with mutations in three (miR-145) or four (-26a, -34a, let-7b) nucleotides in the 5' seed-binding region of the MRE (see Supplemental Table I for sequences). Mutations were screened *in silico* (RNAHybrid) in the context of the full 3'UTR to avoid inadvertent introduction of a novel consensus sequence for another miRNA. Wild-type or seed-mutated MREs corresponding to the four miRNAs were then inserted downstream of a fluorescent reporter. These constructs and normalization controls were transfected into HEK-293T cells. MRE-containing constructs were silenced more efficiently than constructs with seed- mutated MREs (Figure 2B) for all but the miR-145 MRE constructs. This effect was significant ($p<0.05$) for the miR-34a MRE. The lower level of significance for the -26a and let-7b MREs and the opposite result for the miR-145 constructs suggest several possibilities: the mutations we introduced did not fully abrogate binding of the targeting miRNAs; additional native miRNAs may bind to the unmutated regions of the MREs; and/or the introduced mutations may have created

seed binding regions for additional miRNAs that were not predicted by our screening methods. In addition, comparing the results from Figure 1A and B, additional sites for one or more of the four predicted miRNAs present in the full-length UTR may not be represented in the MREs we examined in these experiments.

miRNA mimics reduce, while miRNA antagonists increase, stimulated secretion of IFNβ protein

The potential effect of the four identified miRNAs on IFNβ protein production was assessed in primary human macrophages. Human macrophages were chosen for these experiments for several reasons. As described above, the near-identities of known, expressed macaque and human miRNAs and their respective IFNβ 3'UTRs suggest that these miRNAs play the same regulatory roles in humans and macaques. Additionally, no IFNβ ELISAs we tested could reliably detect macaque IFNβ protein in our hands. For human protein, the ELISA we used was sensitive from about one through several hundred IU/ml with a slightly modified protocol (see Materials and Methods), and the human IFNβ response to poly I:C quickly reached the upper end of this range (Figure 3). Although macaque macrophages exhibit a robust IFNβ response as measured by qRT-PCR and downstream products of the IFNβ signaling pathway [7], only low levels of protein (in the 1-10 IU/ml range) were detected by ELISA in our experiments. Because the amino acid sequences of the respective proteins are highly similar, we hypothesize that species-specific glycosylation differences are responsible for the observed lack of sensitivity to macaque IFNβ protein. Indeed, changes in two potential N-glycosylation sites and one O-linked site appear to be among the macaque-human amino acid

differences (glycosylation predictions made by NetNGlyc, http://www.cbs.dtu.dk/services/NetNGlyc/ and GPP, http://comp.chem.nottingham.ac.uk/glyco/, data not shown) [35].

Macrophages were treated or not with two concentrations of an equimolar mixture of miRs -26a, -34a, -145, and let-7b. The levels of transfected miRNAs were confirmed by qRT-PCR comparison of each miRNA in pre- and post-transfection macrophages (data not shown). Poly I:C RNA (50 ug/ml) was added to the culture medium to stimulate production and secretion of IFNβ protein, and protein levels were measured by ELISA (Figure 3). At 24 hours post-stimulation, the level of IFNβ protein secreted by macrophages treated with 10 or 40 nM miRNA mimics (normalized to no-miRNA, poly I:C-treated control) was reduced by approximately 35% and 80%, respectively, compared with stimulated controls treated with a control miRNA.

miRs -26a [36, 37], -34a [38-40], -145 [41], and let-7 family members [42, 43] have reported effects on cell death processes in cancer, and promotion of apoptosis by transfected cells is one explanation for lower IFNβ production by miRNA-transfected cells. Although no excess cell death was observed in the macrophages transfected with miRNA mimics, the short time course of these experiments might have limited detection. Accordingly, we transfected macrophages with miRNA mimics and control RNA (CmiR) and measured cell death at three and 10 days post-transfection by trypan blue exclusion. No differences in live cell counts were observed between untreated cells, transfection reagent-treated, CmiR-, and miRNA-transfected cells (Supplemental Figure 1). Thus, it is unlikely that the apparent miRNA-mediated downregulation of IFNβ protein is due to promotion of apoptosis by the transfected miRNAs. We also conclude that the pro-

apoptotic effects observed in the cancer literature may not triggered by these same miRNAs in the regulatory environment of healthy primary cells.

Beyond cell death, exogenous miRNA mimics could have other unintended indirect or off-target consequences [44], including saturation of the miRNA processing machinery, stimulation of intracellular signaling pathways, and miRNA-mediated up- or down-regulation of transcripts whose products could affect IFNβ production. As a result, we sought to inhibit native levels of the four identified miRNAs using miRNA antagonist oligos that were chemically modified to enhance stability and reduce off-target effects. These antagomiRs bind to their cognate miRNAs and prevent association with target mRNAs.

The transfected antagomiRs, both singly and in equimolar mixture, increased the amount of IFNβ secreted by primary macrophages exposed to poly I:C stimulation (Figure 4). At 20 nM added antagonist, the effects of miR-34a and let-7b antagonists were significant, as were those of an equimolar mixture of the four miRNAs (all, $p<0.05$). Effects of miR-26a and miR-145 approached significance ($p<0.07$). At 100 nM, miR-26a, miR-34a, and let-7b produced significant effects ($p<0.01$), while miR-145 antagonist approached significance ($p<0.06$). We observed an apparent lack of dose-dependence with the equimolar mixture of antagonist, suggesting that a saturation of some native cognate miRNA may occur at the lower concentration.

The antagonist-mediated increased levels of IFNβ protein do not appear to result from increased levels of IFNβ mRNA. Using quantitative real-time RT-PCR and a ΔΔCt normalization method as described previously [4], we compared IFNβ mRNA levels from primary macrophages treated or not with miRNA antagonists and poly I:C. No significant

differences were found between transcript levels in antagonist-treated and -untreated macrophages (R^2=0.0035; Supplemental Figure 2).

A negative feedback mechanism: IFNβ stimulates expression of modulating miRNAs

We next assessed the potential biological significance of these results by profiling miRNA expression in primary macaque macrophages treated or not with recombinant IFNβ. Preliminary results from miRNA microarrays had indicated upregulation of three of the four IFNβ-targeting miRNAs upon stimulation with recombinant IFNβ (data not shown). We used a quantitative and mature miRNA-specific method, stem-loop qRT-PCR [45], to measure the levels of miRNAs in primary macaque macrophages treated or not with recombinant IFNβ. Three of the four miRNAs were upregulated in response to IFNβ, and the results suggest the possibility of distinct temporal regulation patterns for different miRNAs in response to IFNβ (Figure 5). miR-26a was upregulated by two hours post-IFN treatment, and levels continued to increase through eight and 24 hours. In contrast, miR-34a increased by 2 hours, was lower at eight hours, and increased again by 24 hours post-treatment. let-7b initially increased dramatically, but decreased to background levels at subsequent time points. Consistent modulation was not observed with miR-145.

To address whether native IFNβ protein, produced in response to a stimulus such as exogenous dsRNA, could reproduce the effects seen with recombinant protein, we treated primary human macrophages with poly I:C and sampled culture supernatants and total cellular RNA at one, three, eight, and 24 hours post-treatment. ELISA for secreted IFNβ (Figure 6A) revealed that at one hour, protein levels were at or below the limit of

detection and did not differ from those of untreated cells. IFNβ was detectable by three hours; a large increase was observed by eight hours. IFNβ remained elevated through 24 hours post-treatment.

Modulation of the three miRNAs that responded to recombinant IFNβ was measured by stem-loop qRT-PCR (Figure 6B). Increased miR abundance in poly I:C-treated cells, compared with untreated cells, occurred only at or after the first detection of IFNβ protein, providing further evidence that IFNβ mediates the abundance of IFNβ-targeting miRNAs through a negative feedback mechanism. miR-26a follows a steady increase like that elicited by recombinant IFNβ. Similarly, let-7b increases initially and then declines. For both miR-26a and let-7b, early upregulation in the presence of initially low IFNβ protein levels suggests exquisite sensitivity of these miRNAs to IFNβ. The miR-34a response, in contrast, does not appear until much later, when IFNβ protein is present at over 100 IU/ml.

Discussion

Our results suggest that miRNAs -26a, -34a, -145, and let-7b may modulate expression of IFNβ, thereby influencing innate immunity from the earliest responses to viral infection. For -26a, -34a, and let-7b, this modulation may be exerted directly through miRNA recognition elements in the IFNβ 3'UTR. Although we used macaque IFNβ sequences to evaluate direct interactions, our results appear to apply as well to human IFNβ, as supported by ELISA experiments with human macrophages. Based upon target predictions and the sequences of human and macaque IFNβ, we observe that although the human and macaque 3'UTRs have only 94% sequence identity, all but two of the nucleotide differences are found in the relatively MRE-devoid AU-rich 3' half of the UTR. Also, miRNA binding is thought to be determined largely by perfect or near-perfect target complementarity to a "seed" region of six to eight nucleotides at the 5' end of the miRNA, and neither of the two macaque-human nucleotide differences in the 5' half of the IFNβ 3'UTR is found in a relevant predicted seed-binding sequence. The human and macaque miRNA sequences we have examined are identical. Many miRNAs and IFNβ are conserved in vertebrates, implying that the identified miRNA-IFNβ interactions may not be restricted to primates.

The potential biological significance of miRNA regulation of IFNβ in primates receives *in vivo* support from our SIV/macaque model of HIV encephalitis, as three of the four putative IFNβ-modulating miRNAs are upregulated in the CNS at 42 days post-SIV infection (K. W. Witwer, unpublished observations) and may contribute to maintaining the low levels of IFNβ measured at this time point [4]. Moreover, biological significance is also supported by the apparent presence of a negative feedback loop. Three of the

identified IFNβ-modulating miRNAs are upregulated in response to both recombinant IFNβ and poly I:C-stimulated production of native IFNβ in primary macrophages, suggesting that a biological feedback mechanism may govern the interaction of miRNAs and IFNβ: IFNβ triggers production of miRNAs capable of binding the IFNβ transcript and interfering with protein production.

To confirm that IFNβ itself is necessary for upregulation of miRs -26a, -34a, and let-7b, we performed several experiments with neutralizing antibody to IFNβ. Curiously, the neutralizing antibody appeared to potentiate, not abrogate, the IFNβ response (data not shown), a result consistent with a recent report [46] wherein IFN-neutralizing antibodies are shown to elicit or potentiate Type I IFN responses in endothelial cells and PBMCs. The reported effects are dependent on IFN binding and the presence of the Fc portion of the antibody. In light of these results, future experiments using Fab neutralizing antibody fragments may further clarify the role of IFNβ in miRNA regulation.

Since both miR-26a and let-7b are expressed at relatively and constitutively high levels in many cell types, these miRNAs may exert a constant inhibitory pressure on IFNβ levels even in the absence of miRNA upregulation [47].

Of note, the two IFNβ-regulating miRNAs, -26a and -34a, which are increased from two through 24 hours after IFNβ treatment of primary macrophages, are both implicated in cancer and have been studied in the clinic or as potential treatment targets. miR-26a has recently been shown to affect cell cycle progression and used as a therapy for liver cancer in an animal model [36]. It may be modulated diurnally [48], and among its targets are PTEN and the Ezh2 histone methyltransferase [49-52]. miR-34a has been

characterized as a p53-regulated miRNA involved in cell cycle progression and apoptosis [53] and as part of a positive feedback loop involving p53 and SIRT1, a direct target of miR-34a [54]. Like miR-26a, -34a is implicated in cancers, and its expression has been studied in a clinical trial of chronic lymphocytic leukemia [55]. Our results indicate an additional mode of action for these important miRNAs.

The mechanisms governing transcriptional and post-transcriptional regulation of IFNβ-regulating miRNAs demand experimental study, but we note here that the genomic context of the primary transcripts for miR-26a, miR-34a, and let-7b suggests several regulatory strategies. The start sites and promoters for these transcripts are found in or near CpG islands [56, 57], implying epigenetic control. Numerous predicted and several experimentally validated transcription factor binding sites are also present and could contribute to regulation.

Of particular interest are two transcription factors, p53 and Stat3. The connection between IFNβ and p53 signaling has recently been characterized [58]. miR-34a [38] is a reported p53 transcriptional target, and the let-7b coding region contains a p53 binding site [56]. It is thus possible that p53 mediates the IFNβ-stimulated upregulation of these two miRNAs.

We further propose that rapid downregulation of let-7b, following a brief post-IFNβ surge, may be effected by Stat3, a transcription factor involved in interferon signaling. Stat3 has been reported as an activator for miR-21 expression [59], but a single transcription factor can have opposite effects in different settings [60]. Stat3 has been implicated as a negative regulator of IFN-mediated antiviral responses [61] and, reminiscent of its reported role in downregulating E-Cadherin expression [62], Stat3 may

be responsible for repressing let-7b following initial IFNβ-mediated transcriptional upregulation. Interestingly, the predicted Stat3 binding site is actually within the sequence encoding the mature let-7b.

Regulation of innate immune responses, and particularly of IFNβ, has been demonstrated to occur at multiple levels; however, miRNAs have not previously been implicated directly in this regulation. We demonstrate for the first time that secretion of IFNβ from primary macrophages is reduced by miRNAs that recognize sequences in the 3'UTR of the mRNA.

The regulation of IFNβ by miRNAs as demonstrated in these studies is consistent with our broad hypothesis that a cytokine pivotal in downstream signaling for innate immunity requires regulation at every level of expression. IFNβ is among the first genes induced in response to, e.g., retroviral infection, and it then regulates expression of signaling pathways, transcriptional regulation, and the induction of antiviral cascades. Two- to four-fold changes in the level of IFNβ mRNA and protein initiates signaling cascades that ultimately magnify the IFNβ effect thousands to ten thousands-fold. Because these downstream changes include potent inflammatory cytokines, the cell has evolved highly sensitive controls, both positive and negative [63], to keep them in careful balance, and previously unknown mechanisms continue to be discovered [64]. miRNA regulation of the IFNβ protein is now added to transcriptional control, message stability, and protein stability as an effector of IFNβ regulation.

We note that the miRNAs reported here are unlikely to be the only miRNA species to affect the IFNβ 3'UTR, for at least two reasons. First, despite considerable advances and much thoughtful work in the development of prediction algorithms, no

single method or combination of methods is yet completely reliable. Second, we observe that most studies in the current literature make the same assumption that guided our selection of candidate IFNβ regulatory miRNAs: namely, that regulation of a message by a small RNA presupposes (or is suggested by) regulation of the miRNA itself. This is probably an oversimplification. Modulation of gene expression by miRNA is unlikely to stand alone, but, rather, is probably part of a constellation of regulatory mechanisms. A change in any one of the parts of this system could place a greater burden on another arm of the system without corresponding up- or downregulation of the respective components. Since miRNA-mediated regulation without differential expression of the regulating miRNAs is passed over by candidate screening, such as in our methods, it is possible that additional small RNA regulators of IFNβ await discovery.

With an increasing number of innate immune system components showing evidence of miRNA targeting, the demonstrated potential of miRNA-based therapeutics [36, 65] provides a promising new possibility for modulation of the body's first line of defense against viral infections.

Materials and Methods

MicroRNA target predictions and in silico genomics

Sequences of known and predicted mature human and macaque miRNAs were obtained from the microRNA Registry at miRBase, http://www.mirbase.org/ [66-68]. IFNβ sequences were from the NCBI Nucleotide database, http://www.ncbi.nlm.nih.gov/. The prediction algorithms miRanda [28], RNAhybrid [29], microInspector [30], and PITA [31] were used to search for miRNA target sites in the macaque and human IFNβ 3'UTRs. Where applicable, miRNA-target seed sequence matches were allowed to include G:U wobble and up to one mismatch. Human and macaque IFN-beta proteins were found to be 95% identical and 97% similar by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi for NP_001129267.1 and NP_002167.1). miRNA primary transcript sequences were obtained from the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/) [56]. The presence of CpG islands and transcription factor binding sites was assessed with the UCSC browser tracks "CpG Islands" [57] and "HMR Conserved Transcription Factor Binding Sites" by Weirauch and Raney.

Cells and Reagents

Monocyte-derived macrophages were isolated from pigtailed macaques (*Macaca nemestrina*) and from human subjects as previously described (7). The Johns Hopkins Institutional Review Board reviewed and approved all human and animal studies, and all samples were obtained in accordance with IRB protocols. Human cells were used in experiments involving ELISA quantitation of IFNβ protein, as the ELISA reagents

available are relatively insensitive to macaque IFNβ (in our hands). HEK-293T cells were acquired from the America Type Culture Collection (ATCC, Manassas, VA). miRNA mimics and antagonists were purchased from Ambion (Austin, TX) or Dharmacon/Thermo Fisher Scientific (Waltham, MA). Negative control RNA was the miRIDIAN microRNA Mimic Negative Control #1 from Dharmacon (Sequence: UCACAACCUCCUAGAAAGAGUAGA), screened against the human and macaque genomes by using the BLAST-like alignment tool [69]. Oligonucleotides for cloning were obtained from IDT (Coralville, IA; see Supplemental Table I for sequences). Recombinant IFNβ (PBL-Interferon Source, Piscataway, NJ) and polyinosinic:polycytidylic acid (poly I:C, Amersham/GE Healthcare, Piscataway, NJ), were used at 100 U/ml and 50 µg/ml, respectively.

RNA isolation

Total RNA was isolated and purified by the Trizol method (Invitrogen, Carlsbad, CA) or miRvana kit (Ambion) according to the manufacturer's protocol. Concentration and purity were measured using a NanoDrop spectrophotometer (ND-1000 with V3.5.2 software, Thermo Fisher Scientific) and denaturing RNA gels, which were imaged by an Eagle Eye detection system (Stratagene, La Jolla, CA).

Plasmid vectors

The 3'UTR of the pigtailed macaque IFNβ mRNA was generated by PCR and inserted between the XhoI and NotI restriction sites downstream of the *Renilla* luciferase gene of the dual luciferase vector psiCHECK-2 (Promega, Madison, WI). Sense

oligonucleotides containing the predicted wild type and mutated microRNA recognition elements of the IFNβ 3'UTR (for miRNAs let-7b, miR-26a, miR-34a, and miR-145) were annealed with corresponding antisense oligos and inserted between the XhoI and KpnI restriction sites downstream of the in pEGFP-C1 (BD Clontech, Palo Alto, CA). Oligo sequences are provided in Supplemental Table I.

Luciferase assays

HEK-293T cells were co-transfected with psiCHECK-2 (with or without the IFNβ 3'UTR) and miRNA mimics using Lipofectamine 2000 (Invitrogen). Cell lysates were prepared 24 hours post-transfection and luciferase levels were measured using an Ascent Fluoroskan fluorometer and the Dual Luciferase Reporter Assay System (Promega).

Fluorescence assay

HEK-293T cells were cotransfected with specific pEGFP-C1 constructs and a transfection control, pdsRed-N1 (BD Clontech). Fluorescence expression was measured 24 hours later with a Typhoon scanner (Amersham/GE Healthcare, Piscataway, NJ) and analyzed by ImageQuant software (Amersham). For each transfection, green fluorescence intensities were normalized by red fluorescence.

IFNβ ELISA

Human macrophages were transfected with miRNA mimics or antagonists (Dharmacon, Qiagen, or Ambion) using HiPerFect (Qiagen). After six hours, cells were washed with PBS and refed with media containing 50 µg/ml poly I:C. Culture

supernatants were collected after 24 hours, and IFNβ levels were measured by ELISA (FujiRebio - Invitrogen) according to the manufacturer's protocol but with an overnight primary incubation at 4°C to increase sensitivity. ELISA results were obtained using a Microplate Reader, Model 680 (Bio-Rad, Hercules, CA).

miRNA and IFNβ mRNA qRT-PCR

Macaque macrophages were treated with 100 U/ml of IFNβ (PBL), and cells were harvested at 2, 8, and 24 hours post-treatment for RNA isolation. Levels of mature miRNAs were measured by individual qRT-PCR assays (Applied Biosystems, Foster City, CA) per manufacturer's protocol, using 10 ng of total template RNA. Quantitative RT-PCR for IFNβ mRNA was performed as described previously [4].

Statistical analysis

Analysis was performed using Microsoft Office 2004 Excel, including the Data Analysis add-on (Microsoft, Redmond, WA), and GraphPad Prism, version 6 (GraphPad Software, San Diego, CA). For Student's t-test, two-tailed tests were performed and equal variance was not assumed. Confidence intervals were generated using Data Analysis (Excel).

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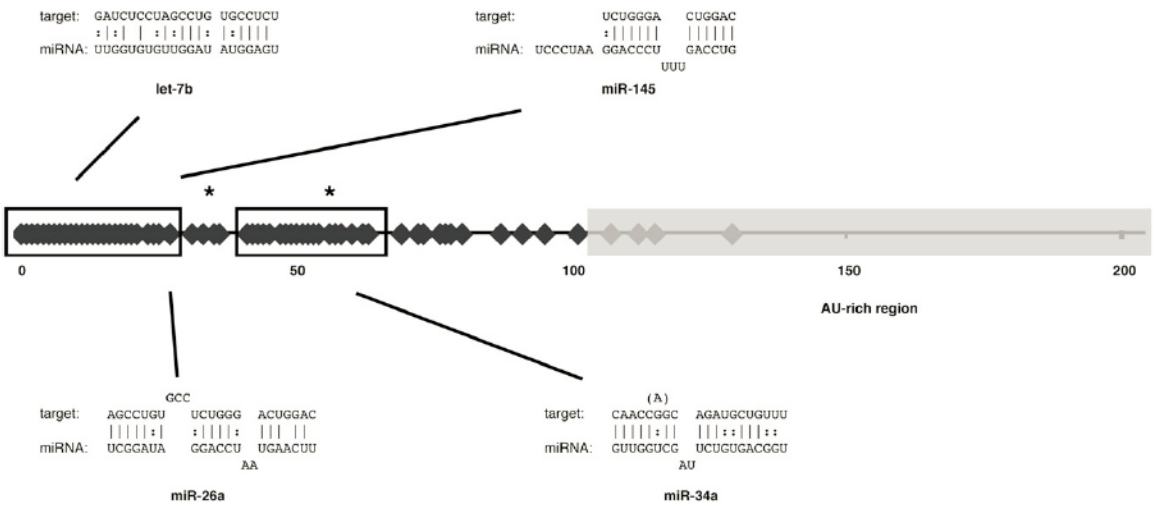


Figure 1. Predicted miRNA recognition elements in the IFNβ 3'UTR.
miRNA prediction algorithms were used to evaluate the likelihood of miRNA binding in the IFNβ 3'UTR. More than 200 hits (3' ends represented as diamonds along the ~200 nt 3' UTR) were filtered by algorithm overlap and expression analysis, with six miRNAs (including the four forming the duplexes shown, which were later validated) selected for further analysis. The 5' half of the 3'UTR contains most of the predicted miRNA recognition elements (MREs), including those for the four selected miRNAs, which are further concentrated in two areas as indicated by black-outlined boxes. The 3' half of the UTR is AU-rich (shaded region) and contains relatively few predicted target sites. MREs for miRs -145, -26a, and -let-7b overlap in the first box, while the predicted -34a MRE starts at nt 50 of the 3'UTR. From human to macaque, only two nucleotide changes are found in the target-rich region (indicated by asterisks). Neither affects seed-binding regions though to be needed for miRNA interaction. Duplexes are shown with the target sequence above the miRNA sequence. G:U wobble (':') and Watson-Crick pairing ('|') are indicated. A macaque to human transition in the -34a binding site (parentheses) makes the human pairing marginally more favorable.

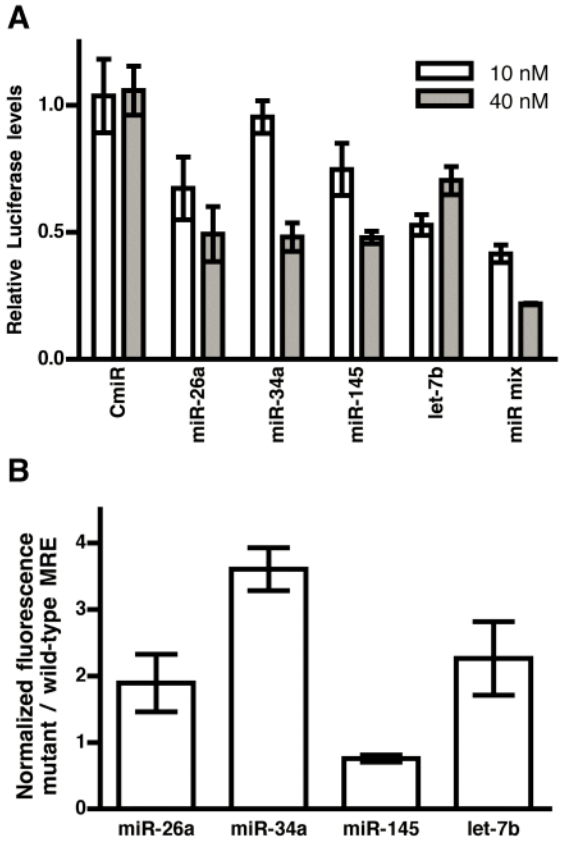


Figure 2. miRNAs directly target sequences in the IFNβ 3'UTR.
miRNA mimics reduce luciferase expression from an IFNβ 3'UTR-containing dual firefly/*Renilla* luciferase reporter vector (A). Normalized luciferase expression is reduced in HEK-293T cells transfected with specific miRNA mimics and equimolar mixtures of the four indicated miRNAs, but not in cells transfected with a control miRNA (CmiR). Each condition is presented relative to a no-miRNA control, set equal to one. Error bars are SEM from three independent experiments. Individual predicted IFNβ miRNA recognition elements cloned downstream of GFP suppressed GFP expression in HEK-293T cells, but three of four mutated MREs relieved this suppression (B). Fluorescence intensity is displayed as the ratio of mutant to wild-type MREs, normalized to red fluorescence (from pdsRed transfection control). Error bars represent SEM.

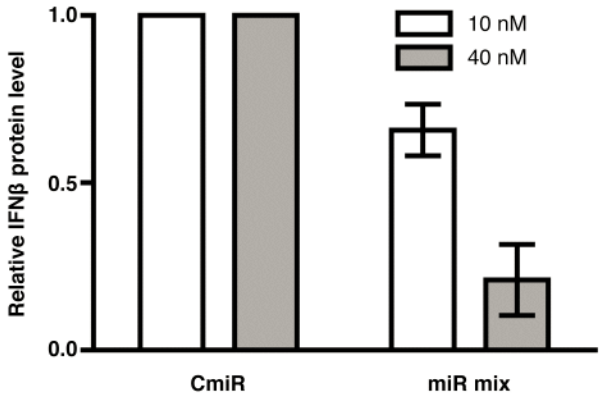


Figure 3. miRNA mimics inhibit IFNβ protein secretion by primary macrophages. A 10 or 40 nM equimolar mixture of four miRNAs (miRs -26a, -34a, -145, and let-7b) or control miRNA was transfected into primary human macrophages, which were then treated with 50 µg/ml poly I:C to stimulate IFNβ production. 24 hours post-treatment, supernatants were collected from no-miRNA controls as well as miRNA- (miR mix) and control miRNA-transfected (CmiR) macrophages; IFNβ levels were measured by ELISA. Levels from control miRNA-treated samples are depicted normalized to poly I:C-treated, no-miRNA controls. Error bars indicate standard deviation.

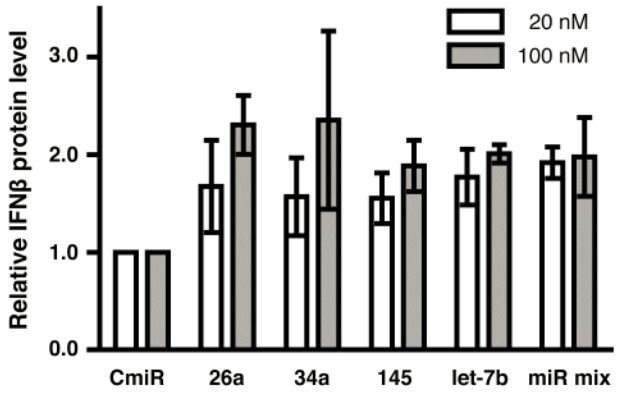


Figure 4. miRNA antagonists relieve native miRNA inhibition of IFNβ secretion. Primary human macrophages were transfected with specific or control antagomiRs, chemically modified to enhance stability and to hinder recognition by intracellular RNA sensors and subsequent activation of the interferon pathway. Macrophages were treated with poly I:C (50 μg/ml). After 24 hours, supernatants were collected and secreted IFNβ protein levels were measured by ELISA. Results from four independent experiments with macrophages from three donors are shown relative to IFNβ levels of poly I:C treated, no-miRNA controls. Error bars indicate standard deviation.

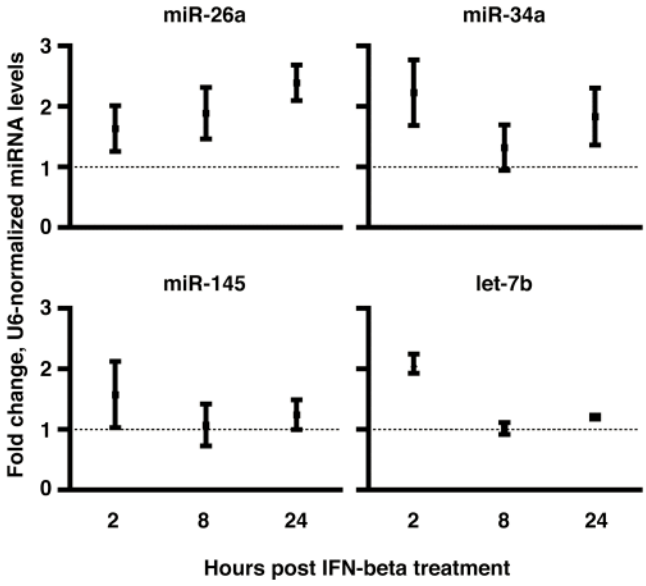


Figure 5. IFNβ treatment of primary macrophages modulates three of four IFNβ-regulating miRNAs.
Primary macrophages from two pigtail macaque donors were treated with 100 U/ml recombinant IFNβ, with RNA collected at two, eight, and 24 hours post-treatment. miRNA levels for miRNAs -26a, -145, -34a, and let-7b were measured on all samples in triplicate by stem-loop qRT-PCR, including no reverse transcriptase and no template controls. The results were analyzed by ΔΔCt, with normalization to U6 snRNA levels and untreated controls. Error bars indicate standard deviation.

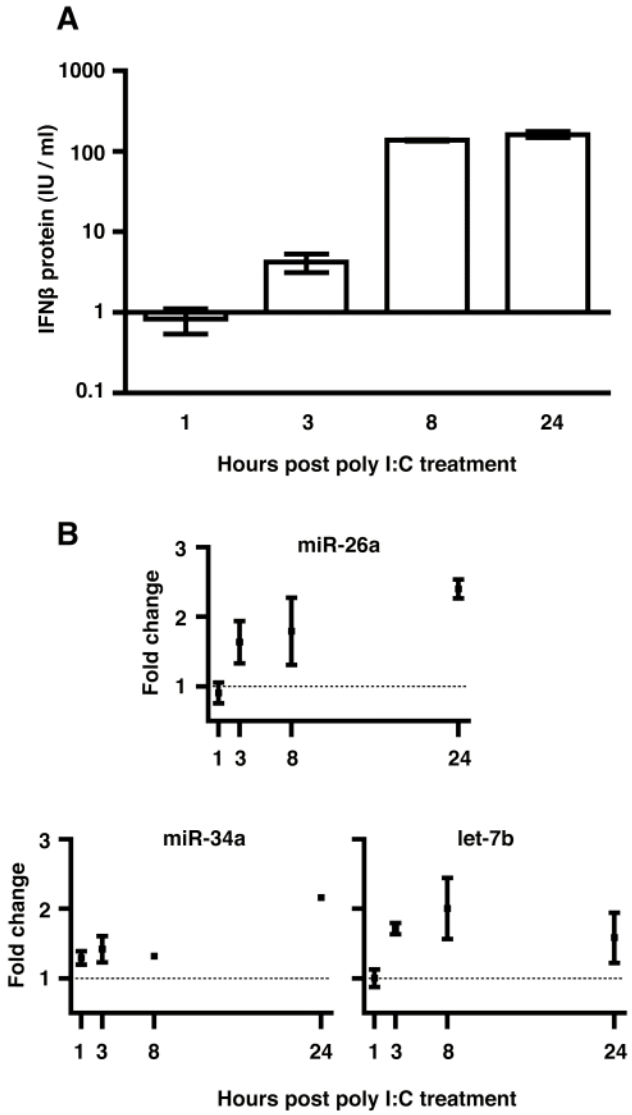
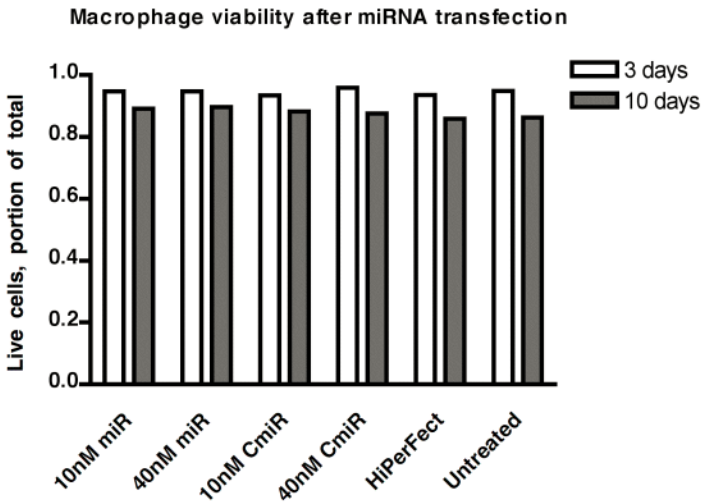
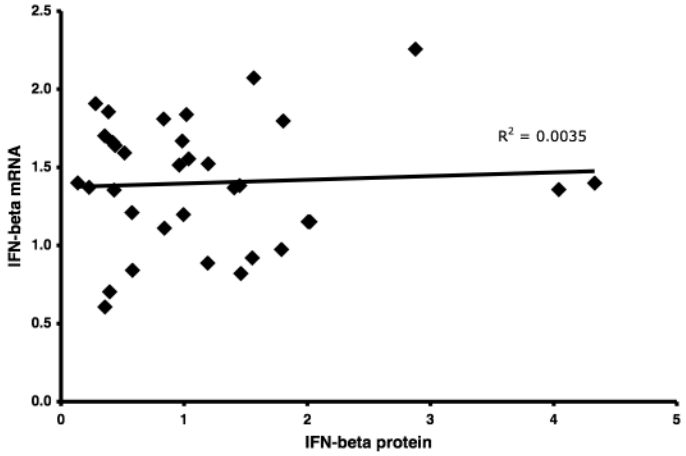


Figure 6. Poly I:C treatment of macrophages induces IFNβ and miRNA production consistent with IFNβ-mediated miR upregulation.

Primary human macrophages were treated with 50 µg/ml poly I:C. IFNβ response (A) was detectable by ELISA by three hours and increased through 24 hours post-treatment. Limit of detection (about 1 IU/ml) is shown as a line. Results are from two donors, measured in duplicate. miRs -26a, -34a, and let-7b were quantified by stem-loop qRT-PCR (B). Results are fold change of treated over untreated macrophages at each time point, normalized to U6 snRNA. Error bars indicate standard deviation.



Supplemental Figure 1. Macrophage viability is unaffected by miRNA transfection. Primary macrophages were transfected with 10 or 40 nM miRNA mixture (miRs -26a, -34a, -145, and let-7b), 10 or 40 nM miRNA control (CmiR), treated with transfection reagent alone (HiPerFect), or left untreated. Viability was assessed by trypan blue exclusion at days three and ten.



Supplemental Figure 2. miRNA antagonist effects on IFNβ protein are not due to changes in IFNβ transcript levels.

Primary human macrophages (treated as described in the legend to Figure 3) were harvested at 24 hours after treatment with 50 μg/ml poly I:C. RNA was purified from all samples treated with poly I:C and transfected with antagonists or control anatagonist. IFNβ mRNA levels were measured by qRT-PCR and results were analyzed by the ΔΔCt method, with normalization of antagonist-treated sample levels to 18S rRNA levels and antagonist control-treated cells. IFNβ mRNA levels were compared with corresponding supernatant protein levels as determined by ELISA (Figure 3). There was no apparent correlation between mRNA and protein levels (R^2=0.0035).

Supplemental Table I. Oligonucleotides.

3'UTR cloning
IFNB3F ATAGCGGCCGCAGATCTCCTAGCCTGTGC
IFNB3R CATCTCGAGTGAATTATGCATCAAAAATAATTTATT
The IFN 3'UTR was amplified from *M. nemestrina* genomic DNA by PCR, digested with Xho I and Not I, and inserted into psiCHECK-2.

3'UTR individual MREs
26a MRE-F TCGATAGCCTGTGCCTCTGGGACTGGACGATATCGTAC
26a MRE-R GATATCGTCCAGTCCCAGAGGCACAGGCTA
26a Mut-F TCGATAGCCTGTGCCTCTGGGAGTGCTGGATATCGTAC
26a Mut-R GATATCCAGCACTCCCAGAGGCACAGGCTA

145 MRE-F TCGACTCTGGGACTGGACAGATATCGTAC
145 MRE-R GATATCTGTCCAGTCCCAGAG
145 Mut-F TCGACTCTGGGACACCTCAGATATCGTAC
145 Mut-R GATATCTGAGGTGTCCCAGAG

34a MRE-F TCGATCAACCGGCAGATGCTGTTTGATATCGTAC
34a MRE-R GATATCAAACAGCATCTGCCGGTTGA
34a Mut-F TCGATCAACCGGCAGATCGACATTGATATCGTAC
34a Mut-R GATATCAATGTCGATCTGCCGGTTGA

7b MRE-F TCGAAGATCTCCTAGCCTGTGCCTCTGATATCGTAC
7b MRE-R GATATCAGAGGCACAGGCTAGGAGATCT
7b Mut-F TCGAAGATCTCCTAGCCTGTGGGAGTGATATCGTAC
7b Mut-R GATATCACTCCCACAGGCTAGGAGATCT

Oligos were annealed and inserted into pEGFP-C1 digested with XhoI and KpnI; a unique EcoRV site was placed into the insert to facilitate screening. All constructs were verified by sequencing.

III. miRNA Profiles of Monocyte-Lineage Cells Are Consistent with Complicated Roles in HIV-1 Restriction

miRNA Profiles of Monocyte-Lineage Cells Are Consistent with Complicated Roles in HIV-1 Restriction

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Abstract

Long-lived HIV-1 reservoirs include tissue macrophages. Monocyte-derived macrophages are more susceptible to infection and more permissive to HIV-1 replication than monocytes for reasons that may include the effects of different populations of miRNAs in these two cell classes. Specifically, miRs-28-3p, -150, -223, -198, and -382 exert direct or indirect negative effects on HIV-1 and are reportedly downmodulated during monocyte-to-macrophage differentiation. Here, new experimental results are presented along with reviews and analysis of published studies and publicly available datasets, supporting a broader role of miRNAs in HIV-1 restriction than would be suggested by a simple and uniform downregulation of anti-HIV miRNAs during monocyte-to-macrophage differentiation. Although miR-223 is downregulated in macrophages, other putatively antiviral miRNAs are more abundant in macrophages than in monocytes or are rare and/or variably present in both cell classes. Our analyses point to the need for further studies to determine miRNA profiles of monocytes and macrophages, including classic and newly identified subpopulations; examine the sensitivity of miRNA profiling to cell isolation and differentiation protocols; and characterize rigorously the antiviral effects of previously reported and novel predicted miRNA-HIV-1 interactions in cell-specific contexts.

Introduction

A challenging obstacle to eradication of HIV is the latent reservoir: long-lived cells harboring relatively quiescent integrated HIV. The ability to identify and clear these reservoirs will form the basis for effective, curative strategies [1–4]. The best characterized reservoir is the resting $CD4^+$ T-cell [5,6]. However, multiple macrophage populations in tissues are also important reservoirs [7–9]. While HIV-1 infection and replication is restricted in monocytes, permissivity increases as monocytes differentiate into macrophages [10,11]. The mechanisms underlying this difference are incompletely understood, but one proposed component is the microRNA complement of macrophages, which has been reported to diverge from that of monocytes [12–14].

The details of the monocyte-to-macrophage miRNA divergence in relation to HIV-1 replication, *i.e.*, which miRNAs are differentially regulated, and in what direction, have been a matter of interesting and potentially conflicting results. In this paper, we first assess the level of concordance and discordance between the various publications examining miRNA profiles during monocyte-to-macrophage differentiation. We then present a new set of miRNA profiling data that includes biological replicates of primary monocytes and macrophages from three human donors. Together, these findings are assessed in relation with previously published work and other publicly available datasets to derive conclusions about the consequences of monocyte differentiation-related miRNA regulation for HIV-1 replication and to identify important questions for continuing research in this area.

In 2009, X. Wang *et al.* reported a pronounced downregulation of several putatively anti-HIV-1 miRNAs as monocytes differentiated into macrophages [13]. The

authors suggested that miRNAs that are abundant in monocytes act to inhibit HIV-1, and that when levels of these miRNAs are reduced during differentiation into macrophages, HIV replicates more productively. In contrast, Coley *et al.* reported no downmodulation of these or other miRNAs in macrophages compared with monocytes [15]. Dicer, the major cytoplasmic miRNA processing enzyme [16], was not detected in monocytes, allowing only limited miRNA production through the PIWI alternative processing pathway [15,17]. Differentiation of monocytes into macrophages was accompanied by Dicer production and concomitant increases in miRNA levels [15,17]. Coley *et al.* posited that relief of HIV-1 restriction in the presence of larger amounts of miRNAs in macrophages could be achieved through repressive actions of viral proteins (Vpr, Nef, Tat) on Dicer. Coley *et al.* did not report differential regulation under any conditions—differentiation or HIV-1 infection—of any of the miRNAs reported to be downregulated by X. Wang *et al.* However, it is unclear that definitive conclusions should be drawn from these apparent contrasts, since the global miRNA profiling in the Dicer study [15] was done using PMA-induced differentiation of the monocytic U937 line, while X. Wang *et al.* examined four miRNAs in primary cells [13].

Profiling studies of PMA-induced cell line differentiation models offer important points of comparison to these HIV-1-focused studies. In 2011, a hybridization study of miRNA profiles before and after PMA-induced U937 differentiation was published by J. Wang *et al.* [18]. Biological triplicates allowed statistical analysis, dye swap experiments for two replicates permitted elimination of artifacts based on dye bias, selected results were confirmed by individual qPCR reactions, and the authors reported their raw data and methods per MIAME requirements [19,20]. Of 44 differentially regulated miRNAs, 12

were downregulated in differentiated U937 cells. Of the 32 upregulated miRNAs [18,20], approximately ten (see Table 1) were found among the 64 upregulated miRNAs reported by Coley *et al.* [15]. Additionally, two putative anti-HIV miRNAs were up-, not downregulated. Li *et al.* included qPCR evidence for significant downregulation in the U937 system of miRs-15a, -16, and -223, but only slight changes in miR-142-5p or let-7 family members [21]. Using another differentiation model—PMA stimulation of THP-1 cells—Forrest *et al.* performed hybridization microarrays for three biological replicates at a zero hour time point and at several time points post-PMA treatment; next generation sequencing was also done, and the data were deposited with CIBEX [22–24]. At 96 hours post-PMA treatment, 23 miRNAs were differentially regulated by three-fold or more. Following PMA treatment of the HL-60 line, Chen *et al.* [25] and Kasashima *et al.* [26] also observed differential regulation.

The results of our comparisons of these experiments are listed in Table 1. We posit that judicious comparison of these results is feasible despite differences in specific myeloid line, PMA concentration, and differentiation time. PMA concentrations (16–300 nM) were within the relatively wide range customarily employed in these models, and although RNA was collected at time points from 24 to 96 hours, differential expression of miRNAs begins within hours of PMA treatment and remains largely constant from 24 to 96 hours in the THP-1 model [22]. Thus, although culture conditions may very well affect results, commonly regulated miRNAs may be considered robust correlates of differentiation in these models.

The first miRNA profiling of primary monocyte-to-macrophage differentiation was reported in 2007 by Fontana *et al.*, who generated monocytic cultures from adult

$CD34^+$ hematopoietic progenitor cells and differentiated monocytes into mature macrophages in the presence of macrophage colony stimulating factor (M-CSF) [12,27]. As confirmed by quantitative real-time PCR and Northern blots, miRs-17, -20a, and -106a (components of the miR-17/92 clusters) were downregulated during differentiation of unilineage monocytic cultures [12]. Members of this group also reported evidence for involvement of miR-424 in the monocyte-to-macrophage differentiation process [28]. Fontana *et al.* cited unpublished microarray studies that formed the basis of their work. There do not appear to have been subsequent publications or database submissions based on this dataset, which would certainly be a valuable addition to the available evidence on the role of miRNA in monocyte-to-macrophage differentiation.

Indeed, to our knowledge, the only monocyte-to-macrophage differentiation miRNA study to date that has examined primary cell profiles with biological replicates, global miRNA profiling, and PCR verification was presented by Sung and Rice in 2009 [14]. These investigators, like most teams that examined cell lines, did not find uniform up- or downregulation of miRNAs. Rather, after gathering hybridization microarray profiles of monocytes and MDM derived from two human donors, they reported that, while most miRNAs maintained relatively constant expression, there were several examples of differential regulation in either direction (nine up and thirteen down) [14]. Interestingly, these results also confirmed one of the four downregulated miRNAs (miR-223) reported by X. Wang *et al.* in primary cells [13], while suggesting that another, miR-150, might be upregulated in some macrophages.

Results and Discussion

2.1. Differential Regulation of miRNAs: New Evidence

The disparities in the published results in the HIV-1 field and in the general monocyte differentiation literature on cell lines and primary cells prompted us to conduct further profiling studies with monocytes and monocyte-derived macrophages from human donors. We began with an experiment using cells from two donors (labeled throughout as donors I and II), performing anti-CD14 bead-based isolation of monocytes from PBMCs. Isolated monocytes were >98% pure and viable as assessed by flow cytometry. Total RNA was isolated from the isolated monocytes. At the same time, PBMCs from the same donors were differentiated into monocyte-derived macrophages (MDM) for seven days [29]. Total RNA was then purified from MDM. To minimize the possibility of dye-related artifact (Cy3 signals are often slightly stronger than those for Cy5, and Cy5 results are disproportionately affected by environmental conditions such as atmospheric ozone levels [30,31]), Cy3 and Cy5 dye-swap hybridizations were performed for each sample with hybridization microarrays. Because microarray experiments are also susceptible to batch effects [31,32] and may thus include artifactual elements [33], we repeated the experiment several months later with cells from a third donor to assess the robustness of results from different batches. Finally, we performed the same differentiation with cells from a leukopack that was shipped to our laboratory overnight; at least 24 hours elapsed between the initial blood draw and isolation of PBMCs and monocytes. We have previously observed that cell activation states differed between shipped leukopacks and freshly obtained blood [34], and we wished to observe

whether any differential expression was sufficiently robust to be seen in leukopack-derived cells, as well.

Results are presented in Figure 1, Table 2, and on a miRNA-by-miRNA basis below. Many of our results are consonant with previous findings (Figure 1 and Table 2, Group I [14]), despite several differences between our cells and culture conditions and those used in other studies, but we also find evidence for differential regulation of miRNAs heretofore unreported in monocyte-to-macrophage differentiation (Table 2, Groups II and III). We focus initially on miRNAs that have putative direct or indirect anti-HIV-1 roles; the genomic neighborhoods of these miRNAs are presented in Supplementary Table 1.

2.2. miR-29 Family

miR-29a is the only miRNA reported by at least two groups to have a direct effect on HIV-1 expression [35–37] on the basis of reporter assays in which HIV-1 sequences were included in a reporter plasmid and exposed to miRNAs, including conclusive evidence of RNA-RNA interaction from experiments in which the putative target site was mutated [36]. miR-29a is encoded with miR-29b in one transcript on chromosome 7, while another copy of miR-29b is co-transcribed with miR-29c from a cassette on chromosome 1. Because miR-29b and miR-29c share an identical seed sequence and are otherwise highly similar to miR-29a, it is likely that all family members would exert some effect on HIV. Along these lines, Ahluwahlia *et al.* presented evidence for direct regulation by miR-29b [35], while Chiang *et al.* reported indirect influence through miR-29b-mediated regulation of Cyclin T1 [38]. Finally, our group has shown that miR-29

family members interact directly with simian immunodeficiency virus (SIV) in macrophages [39,40] by means of reporter/mutation and functional assays.

In our studies, miR-29a was more abundant in macrophages than in monocytes for cells from all donors we examined, including cells from a leukopack that was shipped overnight and processed at least 24 hours after the initial blood draw (Figure 1, Table 2). Similar upregulation was previously reported by in primary cells [14] and during PMA-induced differentiation of myeloid leukemic cell lines [15,18,23] (Table 1). Like miR-29a, miR-29b is upregulated during monocyte-to-macrophage differentiation. However, miR-29b is generally present at lower copy numbers than miR-29a, and low signal intensity precluded a definitive conclusion of differential expression in cells from the third donor. Upregulation of miR-29b was also observed in monocyte-to-macrophage differentiation of cells from the leukopack.

2.3. Ant-HIV-1 miRs-28-3p, -125b, -150, -223, and -382

HIV-1 3' LTR site-specificity of miRs-28-3p, -125b, -150, -223, and -382 was demonstrated using reporter/mutation assays as described above [44]. The authors of this report ascribed to these miRNAs a role in mediating HIV-1 restriction in resting *versus* activated T-cells [44]. To date, direct interactions of these miRNAs with HIV-1 do not appear to have been confirmed by independent researchers; however, anti-HIV effects of some of these miRNAs were later reported in monocyte-derived cells [13] or during drug treatment of monocyte-derived cells [45,46]. It was reported that the levels of four of five of these miRNAs—miRs-28, -150, -223, and -382, as measured by qPCR—fell dramatically (in some cases by more than ten-fold) during differentiation of monocytes

into macrophages [13] and concluded that downregulation of these miRNAs likely enables permissivity to HIV-1 replication in macrophages.

However, there are several potential complications with this interpretation. First, in a response by Swaminathan *et al.* [47], it was argued that the presented data could not support a conclusive statement that the four miRNAs were involved in differential susceptibility of monocytes and macrophages to HIV-1 infection, and that non-HIV targets of these miRNAs must be considered in any analysis of their role in HIV-1 restriction. Second, data normalization methods were not described [13]. In two subsequent publications by the latter group, normalization using a GAPDH assay was described in one paper [45] but not in another [46]. Third, since only downmodulation was reported, with no miRNAs or other nucleic acid molecules shown to remain constant or to increase, it is not clear if the reported declines were associated with monocyte differentiation or, rather, with cell death or changes in cell numbers that would have non-specifically affected miRNA levels. Indeed, with the exception of miR-223, our results have limited agreement with these findings.

miR-223. We confirmed downregulation of miR-223 in macrophages, as previously reported in primary cells [13,14] and in a cell line model [21]. The magnitude of the observed downregulation is also consistent with previous findings.

miR-28-3p. A slight decline was observed for miR-28-3p but the fold changes were small (<1.5), and inconsistent data meant that this result was dependent on analysis method. Our study thus provides no solid evidence for differential regulation of miR-28-3p. In future studies, this miRNA could be quantitated in larger groups of samples to

determine whether or not there is in fact subtle modulation during monocyte differentiation.

miR-125b. Signal for miR-125b did not consistently exceed background in our arrays. Thus, either miR-125b levels were very low in the monocytes and macrophages we examined, or the hybridization platform was insensitive in our hands to miR-125b. We note that an increase in miR-125b was previously observed in cells from one of two donors [14], and that the closely related miR-125a (100% identical with miR-125b in the 5' 13 nucleotides) was strongly enriched in macrophages both previously [14] and in our results described herein.

miR-150. miR-150 was consistently and strongly upregulated in each of our experiments, including the leukopack experiment. Previously, upregulation was reported in one of two donors [14]. In contrast, miR-150 was reported to be downregulated approximately ten-fold in MDM according to Wang *et al.* [13].

miR-382. The quantitation of miR-382 was not possible in our samples, since the fluorescence intensity was below the threshold of detection for miR-382 in all samples except those from the leukopack. Although the presence of very low levels of this miRNA cannot be ruled out, we emphasize that there does not appear to be strong evidence for substantial expression of this miRNA in monocytes or macrophages, much less differential regulation. We re-analyzed several published studies as well as publicly available datasets, which either did not reveal the presence of miR-382 in monocytes (or monocyte-containing cell populations) [48–50] or reported no differential regulation [14,49]. Allantaz *et al.* found no miR-382 in nine of nine monocyte samples measured in

one facility, but they did find low levels in monocytes from two of five donors in a separate profiling experiment conducted in a different laboratory [51].

Thus, for the five miRNAs that were originally published as anti-HIV miRNAs in $CD4^+$ T-cells [44], our findings and the preponderance of evidence from published studies and public datasets support the conclusion that only miR-223 appears to be downregulated during differentiation of monocytes into macrophages and thus consonant with a straightforward role in the relaxation of HIV-1 restriction during monocytic differentiation. miR-150, like the miR-29 family members, is consistently more abundant in macrophages than in monocytes.

2.4. miR-198

miR-198-mediated control of Cyclin T1 contributed to restriction of HIV-1 replication in monocytes, and this control was relaxed with downregulation of miR-198 in macrophages [14]. Examination and/or re-analysis of several published reports and other datasets supports the reported low or negligible macrophage levels of miR-198. Re-analyzing a qPCR array dataset from a study of HIV-1-infected and uninfected PBMC (n = 6 each), we found that after sufficient time in culture to promote monocyte differentiation, miR-198 amplified only sporadically and inconsistently [52]. Similarly, a study of macrophage miRNA populations did not detect miR-198 in macrophages by hybridization microarray [53]. We also found no detectable miR-198 in uninfected (n = 6) or infected (n = 42) thalamus samples from a macaque model of HIV encephalitis (brain includes both resident microglia and perivascular macrophages—data not shown).

To what extent miR-198 may be differentially regulated in monocytes remains an intriguing question. In addition to the work by Sung and Rice, hybridization array

profiling of monocytes and other leukocytes pinpointed low levels of miR-198 in some cell types, including monocytes, with levels varying up to 3.5-fold between different donors [51], and low-level miR-198 expression was found at day zero in a study of monocyte-to-dendritic cell differentiation [49]. However, other evidence has not supported the presence of miR-198 in monocytes. We did not detect miR-198 in the monocytes or macrophages in the work reported here (Table 2b), or in RNA from freshly isolated PBMC from controls or HIV-1-infected individuals, as measured by qPCR or by NanoString miRNA hybridization microarray [54]. Re-analyzing an Affymetrix hybridization array dataset from van Eijsden and Ayoubi [55], we found no appreciable expression of miR-198 in any examined blood cell subtype, including monocytes. Another dataset, from Agilent arrays performed by Murray and Swaminathan [50], included profiles of monocyte miRNAs from eight control and 16 HIV-infected subjects, with inconsistent expression of miR-198, which was also not detected in $CD14^{+}$ monocytes from control or morbidly obese individuals (Exiqon array) [48]. Thus, although it would appear to be amply confirmed that miR-198 is not present, or is present at only very low concentration, in macrophages, there is some evidence that abundance in monocytes may be quite variable. Given the importance of miR-198 for Cyclin T1 regulation in monocytes as established by the Rice laboratory, further work is needed to establish the level and variability of miR-198 expression in monocytes and monocyte subtypes, the possible dependence of successful quantitation on monocyte isolation protocols, and the ability of different profiling platforms to detect and quantitate miR-198 successfully.

2.5. miR-17/92 Cluster

Members of the polycistronic miR-17/92 cluster are transcribed from human chromosome 13, and closely related miRNA clusters are found on chromosomes 7 and X (Supplementary Table 1b) [56]. These miRNAs are related by sequence as well as co-transcription. The chromosome 13 cluster was downmodulated following HIV-1 infection of Jurkat cells [57]. Furthermore, several members of the cluster were found to suppress HIV-1 replication indirectly, through direct regulation of the histone acetyltransferase PCAF [57]. At least one member of the cluster—miR-18a—was predicted to interact directly with HIV-1 [44], although another group reported different results [57].

Almost every member of the three miR-17/92 clusters was downregulated two- to five-fold with monocyte-to-macrophage differentiation: miRs-17, -18a, -19b, -20a, -20b, -25, -92a, -93, -106a, -106b, and -363. Previously, in primary cells, miRs-17, -20a, and -106a were differentially regulated [12], while downmodulation of miR-19b was reported for cells from one of two donors [14]. miR-17 was also the most consistently downregulated miRNA in the various reports on PMA-stimulated cell lines (see Table 1).

2.6. Additional miRNAs — Direct Interactions?

HIV-1 target sites have been proposed for a host of miRNAs in addition to those discussed above (Supplementary Table 2). Hariharan *et al.* used multiple targeting algorithms to predict HIV-1 target sequences in isolates representing several virus clades [37]. Nathans *et al.*, who confirmed the direct miR-29a/HIV interaction, also predicted binding sites in the 3' LTR for eight miRNAs not in the miR-29 family [36]. Schopman *et al.*, recently performed in silico analysis of interactions for 38 miRNAs that were enriched in small particles (including virions) produced by SupT1 cells transfected with HIV-1 provirus [58]. Interestingly, Huang *et al.*, chose for follow-up the five specific

miRNAs mentioned above (-28-3p, -125b, -150, -223, and -382) from among no less than 96 host miRNAs for which they predicted interactions with HIV-1 [44]. It is not clear how the five reported miRNAs were chosen, since these miRNAs did not uniformly have the strongest predicted target interactions (as ranked by free energy) or the largest number of predicted targets. Although the authors stated that 31 miRNAs were downregulated by two-fold or greater in activated *versus* resting $CD4^+$ T-cells, and that all five selected miRNAs were depleted, the results of the microarray experiment they presented in the supplementary material showed that (1) only three of the five selected miRNAs were downregulated by two-fold or more, and (2) miR-382 does not appear to have been detected at all (re-analysis of supplementary data from [44]).

These observations raise the exciting possibility that additional small RNA regulators of HIV-1 remain to be characterized, including miRNAs that are differentially expressed in monocyte-to-macrophage differentiation. Several candidates are indicated in Table 2 ('#'). Of particular interest may be those miRNAs that we found to be downregulated in macrophages—including miRs-103, -107, and -425. However, upregulated miRNAs should not be ignored (see Conclusions). Characterization of previously unknown miRNA-HIV-1 interactions may be best guided in future not by target prediction algorithms, but instead by target enrichment strategies like that used by Althaus and Vongrad *et al.* [59]. Indeed, these investigators reported capture of at least 21 host miRNAs using HIV-1 capture probes in primary cell systems, most of which were differentially regulated in our experiments (Table 2).

While pulldown of host miRNAs could potentially be explained by factors other

than canonical miRNA-target interactions, these results are compelling, and the method will likely continue to be useful for miRNA-virus interaction studies.

2.7. Additional miRNAs — Indirect Effects

Apart from additional members of the miR-17/92 clusters, our results indicate differential regulation of more than ten miRNAs that have not been previously associated with monocyte-to-macrophage differentiation in primary cells (Table 2, Groups II and III) and that do not have previously predicted [60,61] binding sites in HIV-1 sequences. Potential indirect effects of these miRNAs on HIV-1 should be considered, as described for the miR-17/92 cluster and miR-198, above. Some known indirect effects do not appear to factor in the monocyte-macrophage system. A role for miR-27 has been found in $CD4^+$ T-cells [38], but we do not find differential expression of miR-27 family members here. miR-217 promotes LTR transactivation by inhibiting the SIRT1 chromatin modifier [62]; however, we did not detect miR-217 in these cells.

3. Experimental Section

3.1. Cell Isolation and Culture

Total PBMC were isolated from freshly drawn blood from human donors or a leukopack (shipped overnight from New York Blood Center) using a Ficoll gradient. $CD14^+$ monocytes were isolated from total PBMCs using anti-CD14 beads (Dynal) and were assessed by flow cytometry (FACSCalibur, BD Biosciences) as >98% pure and viable. Total PBMCs were plated at 10^7 cells per well in 6-well plates for macrophage differentiation as described previously [29]. Macrophages were differentiated in culture for

7 days in medium containing human serum and M-CSF (R&D), with half re-feeding at day 4.

3.2. RNA Extraction

RNA from monocytes and macrophages was extracted using the Trizol Reagent (Life Technologies) RNA extraction protocol. RNA quality was assessed by spectrophotometry.

3.3. miRNA Microarrays

One microgram aliquots of each RNA sample were poly(A) tailed and labeled with Alexa fluor dendrimers AF3 or AF5 (fluorescing in the green and red channels, respectively), using the NCode Rapid miRNA Labeling Systems (Invitrogen). For each donor, two dye-swap array hybridizations were performed with monocyte and macrophage RNA with NCode Human miRNA microarrays (V3). The NCode V3 is a spotted array on a glass slide. Each probe is a tandem repeat of a sequence corresponding to a target. The slide includes probes to over 700 human miRNA targets and additional small RNA sequences reportedly identified by the producer in deep sequencing analyses. Three replicates of each probe are printed on each slide. Arrays were maintained at 52 °C overnight in Maui mixer stations and covered with Maui chamber slips (SL from BioMicro Systems) for hybridization solution recirculation. Slides were then washed for 15 minutes in each of the following solutions, pre-warmed to 55 °C: 2× SSC, 0.2% SDS; 2× SSC; 0.2× SSC. Slides were scanned with an Axon scanner (3000B) and GenePix Pro software [34]. Gpr files were generated, containing the raw data.

3.4. Analysis

Multiple analysis methods and software tools were employed to assess method-independence of results, in part as described previously [63]. Data from all fresh blood sample experiments was background corrected and thresholded and analyzed using normalization methods based on both median and lowess smoothing using BRB-ArrayTools [42]. The limma package from R/Bioconductor [64] was used to conduct an analysis of data from the fresh blood samples. Data were normalized with the "printtiploess" method. Different normalization methods produced results that differed only slightly from each other, with some changes in rank; results were confirmed by generating RG plots for the data pre- and post-normalization: after normalizing, the red and green density curves overlapped each other almost entirely. Boxplots of normalized data showed similar medians and ranges of values (data not shown). For linear modeling, within-array replicates (three per array) were taken into account with the "guessdups" and "duplicateCorrelation" functions. Empirical Bayes was used to moderate the probability distribution. Unmoderated and adjusted p-values and the B statistic were calculated (the latter is presented in Table 2).

Analysis of dye-swap experiments for all donors, including the leukopack experiment, was also performed with NCode Profiler software [65], with p-values assigned by iteration (10,000 bootstraps). Subsequent analysis was restricted to human miRNAs, since the array contained probes for many additional known and predicted small RNAs. Four inclusion filters for analysis of individual miRNA data were established. First, data were ranked by p-values as provided by NCode Profiler software [65], and miRNA replicates with p-values above 0.05 were eliminated. Second, the presence of at least two of three possible dye-swap normalized ratios was required, *i.e.*,

values above threshold for both spots and both dyes for at least two of three technical replicate sets. Third, satisfaction of the second criterion for each of the first two biological replicates was required for inclusion. Finally, an average absolute fold change greater than 1.5 in donors I and II was required. Throughout, Microsoft Excel and the MultiExperiment Viewer [66,67] provided additional analysis tools.

3.5. Literature and Data Re-Analysis

Publicly accessible data were downloaded from the Gene Expression Omnibus or from other websites as indicated. Re-analyses were conducted in part or in whole as described above.

3.6. Data Accessibility

Raw and processed data for the triplicate studies presented here have been deposited with the Gene Expression Omnibus under accession GSE39905 and are fully MIAME-compliant.

4. Conclusions

A synthesis of our data, re-examination and re-analysis of other datasets, and the small number of verified miRNA-HIV interactions might be taken to suggest that few if any miRNAs that are differentially regulated during monocyte-to-macrophage differentiation are poised to affect HIV-1 replication. Only one miRNA (miR-223) that interacts directly with HIV sequences (and relatively weakly: see Supplementary Table 2), has been consistently found to be reduced in concentration in macrophages compared with monocytes. This single downregulation appears to be greatly outweighed by upregulation of highly abundant HIV-1-specific miRNAs with low free energies of interaction like the miR-29 family and miR-150 (Supplementary Table 2), as well as a lack of evidence for downregulation (or indeed presence) of other previously reported anti-HIV miRNAs.

Nevertheless, we posit that dismissing macrophage-enriched miRNAs as irrelevant for HIV-1 regulation would be a mistake. The notion that the concentration of a specific miRNA must necessarily inversely correlate with the abundance of a single target transcript (or transcript product) is conceptually appealing but perhaps oversimplistic, particularly when different experimental conditions or cell types are compared. Changes that occur during the differentiation of monocytes into macrophages—transcriptional, proteomic, morphological—are sufficiently profound that they may greatly complicate stand-alone interpretation of before-and-after profiling of miRNAs (Figure 2). Since each putative anti-HIV miRNA may have scores or hundreds of host mRNA targets, as well as targets in longer non-coding RNAs, and since the strengths of these interactions and the turnover rates of specific transcripts vary [68], the

regulatory pressure exerted by a given miRNA on HIV-1 would be best predicted within the prevailing transcriptional environment [69]. Thus, enrichment of miR-150 and miR-29a in macrophages does not necessarily mean that these RNAs have no role in HIV-1 restriction in monocytes.

Furthermore, in addition to the cellular balance of targets and specific miRNAs, the overall numbers of HIV-1-interacting miRNAs would have to be considered in an effective assessment of miRNA/HIV-1 regulation. It is unlikely that we know the identities of all miRNAs with regulatory target sites in the HIV-1 genome, or in prominent sequence variants. Recall that Huang *et al.* investigated and validated only five miRNAs out of 96 predicted HIV-1 interactors [44], and that only miR-29a has been confirmed by multiple laboratories to bind directly to HIV-1. More research is needed to confirm previously reported HIV-1 interactors and to identify additional small RNAs that interact with HIV-1.

Advances on the miRNA-HIV-1 targeting questions would ideally be accompanied by larger miRNA profiling studies of cell differentiation and activation than those that have been conducted to date. Stemming from observations we made while conducting the multiple re-analyses presented above, we would like to present several considerations to guide future profiling studies in this field. First, biological replicates are essential in all profiling work, since each represents a different human and captures genetic heterogeneity between individuals. Some studies to date have included only one sample per condition, precluding statistically based conclusions about the underlying biology [70,71]. Second, the technical demands of each profiling system must be taken into account [33,72]. For example, dual-channel microarrays are susceptible to artifact

because one dye may be brighter than the other, necessitating dye-swap experiments. Third, careful data processing and normalization should be performed and described to allow replication. Fourth, valid statistical analysis should include multiple comparison correction when statistical analysis is reported for large datasets [73]. Finally, we urge authors of future profiling studies to maximize the usefulness and impact of their work by depositing MIAME-compliant [19] raw and normalized data with one of the public databases, such as the Gene Expression Omnibus (GEO) [74] or ArrayExpress [75].

As research proceeds, it will be important to study how cell isolation and differentiation protocols affect miRNA profiles, and to examine—in addition to monocytes and macrophages as a whole—the various subpopulations of these cell types that have been described [76–78]. On the first point, we note that our cell culture conditions and those of the closely related study of Sung and Rice [14] employed M-CSF and GM-CSF, respectively, which could account for some differences between our results, while at the same time emphasizing the robustness of the common results. On the second point, further miRNA profiling may aid in defining and characterizing cell subclasses. In this direction, Graff *et al.*, recently contributed a profiling study on differences between untreated MDM and those polarized towards different phenotypes (M1, M2a, M2b, and M2c) [79]. miRNAs identified in this report included miRs-125a, -146a, -155, and -222. Additional profiling studies are needed, along with development of tools for functional studies [80], to address the many outstanding questions in this field. As more transcriptome and miRNA profile datasets are collected rigorously and made available to the community, we can expect new insights into the complex post-

transcriptional regulatory networks that influence HIV-1 replication in monocytes and macrophages.

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Conflict of Interest

The authors declare no conflict of interest.

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miRNA	Wang U937	Coley U937	Forrest THP-1	Chen, Kasashima HL-60
down				
miR-17	x			x
up				
miR-21	x			x
miR-22	x	x	x	
miR-23a/b	x	x		x
miR-24			x	x
miR-26a/b	x	x		x
miR-27a/b	x			x
miR-29a	x	x	x	
miR-29b	x	x	x	
miR-132		x	x	
miR-146a	x		x	x
miR-146b	x	x	x	x
miR-221	x	x	x	x
miR-222	x	x	x	x
miR-424	x		x	x
miR-663	x	x		

Table 1. Commonly reported regulated miRNAs: U937, THP-1, HL-60 differentiation. Results of five studies of PMA-induced U937, THP-1, or HL-60 monocyte differentiation models were compared: Wang *et al.* [18], Coley *et al.* [15], Forrest *et al.* [23], and Chen *et al.* [25], and Kasashima *et al.* [26] (combined). Only miR-17 was reported to be downregulated by more than one group, although all but Coley *et al.* reported downregulated miRNAs. Upregulated miRNAs were reported by J. Wang *et al.* (>30), Coley *et al.* (>60), Forrest *et al.* (>20), and the Chen and Kasashima studies (>10 combined). The 15 miRNAs presented here were found to be upregulated in at least two of the four study groups; miRs-146b, -221, and -222 (boxed) were common to all.

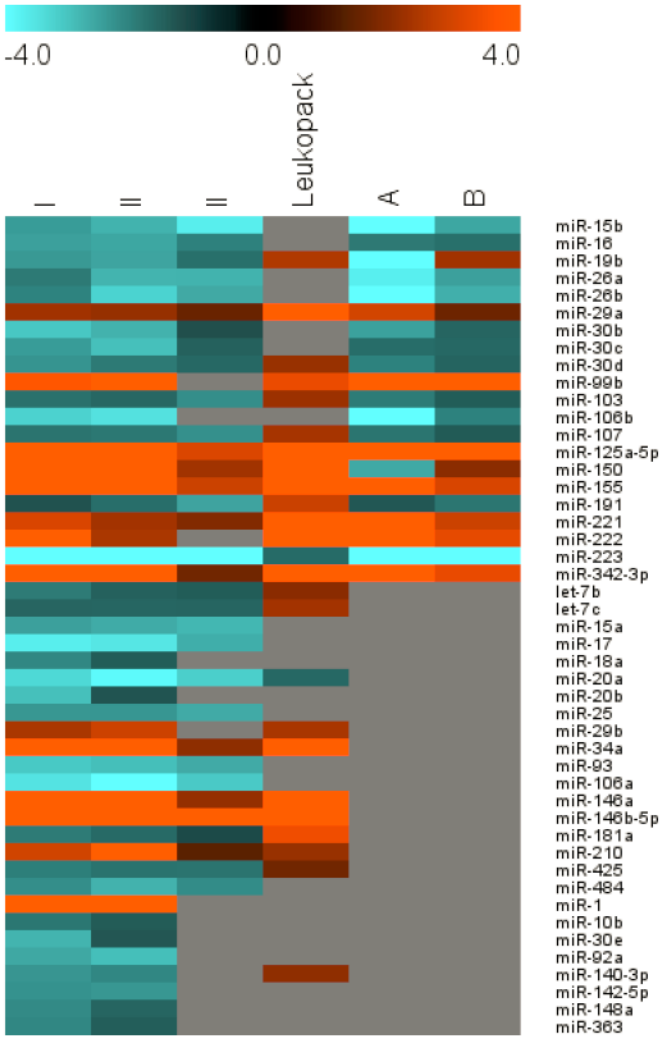

Figure 1. Graphic representation of fold changes for selected miRNAs, comparing monocytes with monocyte-derived macrophages. Positive values indicate enrichment in macrophages over monocytes. Fold changes were calculated for each miRNA from each of three donors: I, II, and III, using normalization by median centering for two dye-swap array experiments for each donor/sample with averaging of three triplicate measurements for each miRNA. Results of a leukopack experiment are also shown. 'A' and 'B' are provided for the sake of comparison: the results of hybridization array analysis performed and reported previously by Sung and Rice and calculated from data obtained from the Rice lab website [41]. Gray indicates that the corresponding miRNA was not detected or not differentially regulated in the respective arrays. See Table 2 for exact fold change calculations, statistics, and results of alternative analysis. MIAME-compliant raw and processed data from the triplicate fresh blood draws are available from GEO as GSE39905; leukopack data are available upon request.

(a)

Group	miRNA	B	Donor I FC		Donor II FC		Donor III FC		Leuko-pack	Sung/Rice FC		Novel inter-action
			MC	DS	MC	DS	MC	DS	DS	A	B	
I	miR-15b	0.37	−2.45	−2.89	−2.80	−3.95	−3.72	−3.05		−6.03	−2.61	
	miR-16	0.82	−2.52	−3.11	−2.61	−3.67	−2.04	−1.72		−1.93	−1.79	
	miR-19b		**−2.42**	**−2.82**	**−2.58**	**−3.13**	**−1.80**	**−2.11**	2.46	***−4.16***	***2.25***	
	miR-26a		−1.95	−2.39	−2.82	−3.92	−2.81	−2.38		−3.72	−2.52	*

Table 2. *Cont.*

Group	miRNA	B	Donor I FC		Donor II FC		Donor III FC		Leuko-pack	Sung/Rice FC		Novel inter-action
			MC	DS	MC	DS	MC	DS	DS	A	B	
	miR-26b		−2.09	−2.56	−3.27	−4.40	−2.64	−2.02		−5.41	−2.73	*
	miR-29a	**0.78**	**2.25**	**1.79**	**2.11**	**1.48**	**1.51**	**1.73**	**5.34**	**2.92**	**1.58**	
	miR-30b		−3.08	−3.72	−2.78	−3.61	−1.29			−2.54	−1.60	
	miR-30c	0.55	−2.46	−2.91	−2.97	−3.77	−1.55			−1.75	−1.67	
	miR-30d		−2.35	−2.74	−1.95	−2.65	−1.67		2.14	−2.07	−1.59	
	miR-99b	3.07	3.73	2.63	4.16	2.51		1.94	3.29	33.77	61.46	
	miR-103		−1.82	−2.21	−1.66	−2.34	−2.27	−1.93	2.18	−1.98	−1.48	#
	miR-106b	0.83	−3.26	−3.78	−3.48	−4.42				−5.08	−2.07	
	miR-107	0.10	−1.86	−2.26	−1.92	−2.73	−2.28	−1.91	2.31	−1.87	−1.45	#
	miR-125a-5p	3.19	6.65	5.59	5.50	6.10	3.05	3.90	6.06	8.91	30.89	#
	miR-150	**1.96**	**5.99**	**4.12**	**14.29**	**8.69**	**2.27**	**2.50**	**5.49**	***−2.66***	***1.99***	
	miR-155	4.54	5.46	4.33	4.86	3.38	2.82	3.11	6.25	8.94	3.00	
	miR-191		−1.33	−1.66	−1.77	−2.46	−2.56	−2.14	2.79	−1.41	−1.89	*
	miR-221	1.97	3.02	2.43	2.28	1.63	1.84	2.06	5.37	6.36	2.81	#
	miR-222	1.32	4.36	3.47	2.37	1.63			5.48	7.26	3.22	#
	miR-223	**0.89**	**−4.23**	**−5.31**	**−6.95**	**−9.88**	**−5.15**	**−4.18**	**−1.72**	**−5.96**	**−5.61**	
	miR-342-3p	5.41	7.01	5.40	6.16	4.23	1.62	1.80	7.39	8.40	3.27	
II	let-7b		−1.96	−2.42	−1.54	−2.17	−1.49		1.97			#
	let-7c		−1.62	−1.97	−1.65	−2.30	−1.60		2.28			#
	miR-15a		−2.52	−3.07	−2.68	−3.65	−2.84	−2.19				#
	miR-17	**0.91**	**−3.71**	**−4.30**	**−3.56**	**−4.50**	**−2.72**	**−2.01**				
	miR-18a		**−2.15**	**−2.25**	**−1.49**	**−2.30**		**−1.63**				#
	miR-20a	**2.61**	**−3.36**	**−4.01**	**−3.89**	**−5.09**	**−3.20**	**−2.52**	**−1.67**			
	miR-20b	**0.01**	**−2.97**	**−3.45**	**−1.36**	**−2.43**		**−1.57**				#
	miR-25		−2.40	−2.84	−2.38	−3.22	−2.67	−2.18				
	miR-29b	**0.89**	**2.37**	**1.81**	**2.83**	**1.79**			**2.37**			
	miR-34a	4.19	10.66	6.97	7.39	6.16	2.02	2.53	5.15			* #
	miR-93	1.65	−3.11	−3.77	−2.96	−4.00	−2.68	−2.26				
	miR-106a	1.99	−3.52	−4.20	−4.01	−5.02	−3.13	−2.61				
	miR-146a	5.75	8.40	6.47	9.75	6.43	2.10	2.24	7.68			*
	miR-146b-5p	5.37	7.50	5.75	11.08	7.20	9.74	10.38	6.88			*
	miR-181a		−1.97	−2.31	−1.70	−2.32	−1.18		3.38			*
	miR-210	1.32	2.89	2.71	4.39	3.88	1.31	1.54	2.14			* #
	miR-425		−2.03	−2.39	−1.85	−2.44	−1.86	−1.78	1.62			#
	miR-484		−2.26	−2.71	−2.79	−3.55	−2.23	−1.85				
III	miR-1	4.90	11.14	7.19	4.98	4.90						
	miR-10b		−1.91	−2.02	−1.46	−1.74						
	miR-30e		−2.81	−2.82	−1.39	−2.62						

	miR-92a	**0.37**	**−2.63**	**−3.17**	**−2.95**	**−4.00**						
	miR-140-3p	0.07	−2.36	−2.59	−2.15	−2.58			2.04			
	miR-142-5p		−2.31	−2.39	−2.39	−2.91						
	miR-148a		−2.21	−2.41	−1.61	−2.13						
	miR-363	1.62	−2.15	−2.89	−1.50	−2.76						

(b)

miRNA	**Donor I**		**Donor II**		**Donor III**		**Leuko-pack**	**Sung/Rice**		**Wang *et al.***
	MC	**DS**	**MC**	**DS**	**MC**	**DS**	**DS**	**A**	**B**	
miR-28-3p	nc	−1.63	nc	−1.65	nc	nc	nc	nr	nr	~−2
miR-125b	bb	bb	bb	bb	bb	bb	bb	nr	1.83	nr
miR-125a-5p	6.65	5.59	5.50	6.10	3.05	3.90	6.06	8.91	30.89	nr
miR-150	6.00	4.12	14.29	8.69	2.27	2.50	5.49	−2.66	1.99	~−10
miR-223	−4.23	−5.31	−6.95	−9.88	−5.15	−4.18	−1.72	−5.96	−5.61	~−10
miR-198	bb	bb	bb	bb	bb	bb	bb	−8.87	−59.18	nr
miR-382	bb	bb	bb	bb	bb	bb	−1.66	nr	nr	~−10

Table 2. Differential regulation of miRNAs in monocyte-to-macrophage differentiation. (**a**) miRNA expression profiles of day zero monocytes and day 7 macrophages were assessed by hybridization microarray in dye-swap experiments with three technical spot replicates per array. Hybridization arrays for Donor I and II arrays were processed together in one batch, while Donor III samples were hybridized several months later to assess reproducibility of results. Also included are results obtained with cells from a leukopack, which was shipped overnight before cell processing (with at least 24 hours between leukapheresis and cell/RNA isolation). Datasets for donors I and II were analyzed following print-tip loess normalization by fitting linear models to the data with limma (R/Bioconductor) and moderating with empirical Bayes smoothing. The 'B' statistic is the empirical Bayes log odds of differential expression, with positive values considered to be statistically significant (corresponding approximately to moderated $p <$ 0.01). Datasets for donors I, II, and III were also analyzed with different methods, with the results displayed under "MC" (high background cutoff, median array centering, using BRBArray-Tools software [42]) or "DS" (Dye Swap, using NCode Profiler software [43]). Calculated fold change values indicate up- (positive, black) or downregulation (negative, red) in macrophages as compared with progenitor monocytes from the same donor. miRNAs included in group I displayed consistent regulation for donors I and II, as assessed by both indicated normalization/analysis methods, with an average FC of 1.5 or greater; plus confirmation in the original Sung and Rice dataset. Italics indicate inconsistent regulation in the two Sung and Rice donors. Group II contains miRNAs that were not reported by Sung and Rice but were confirmed in our experiments for either or both of the third donor or the leukopack. Group III members have evidence for differential regulation only in the datasets for donors I and II and have not been confirmed independently. **Bold** indicates miRNA with reported direct or indirect roles in regulation of HIV-1. Purple values highlight regulation opposite that observed for the majority of datasets analyzed. The "New Interaction" column indicates candidates for novel HIV-1 interactions: miRNAs with published predicted binding sites in the HIV-1

genome that have not yet been confirmed experimentally ('#') or miRNAs that were pulled down with HIV-1 enrichment probes ('*') by Althaus and Vongrad *et al.* (**b**) Fold change for miRNAs with previously reported roles in monocyte-to-macrophage differences in restriction of HIV-1 replication. This table includes data duplicated from 'a' as well as results that did not meet the data filters for 'a'. Data are presented for miRNAs reported by Wang *et al.* [13] or by Sung and Rice [14]. nc = no change, *i.e.*, <1.5 fold change and/or no significant difference in technical replicate groups; nr = not reported; bb = below background. Approximate downregulation (final column) has been estimated from Figure 1 of [13].

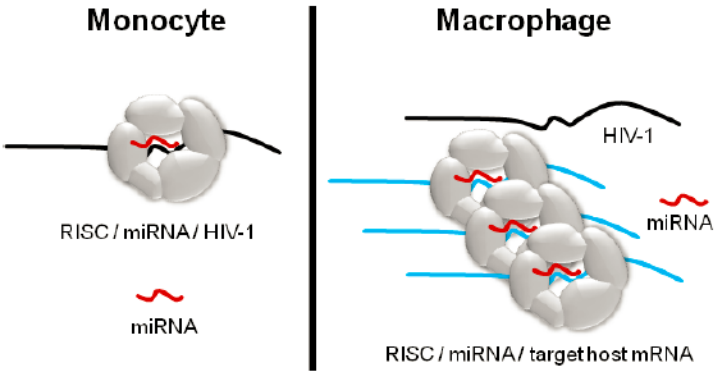

Figure 2. miRNA-mediated regulation depends on factors in addition to miRNA copy number. A hypothetical host miRNA (red) and the RISC machinery binding to the HIV-1 transcript in a monocyte but not in a macrophage. With differentiation of the monocyte, the miRNA is upregulated two-fold, but a host target transcript (blue) with a high-affinity target site for the miRNA is also produced, acting as a "sponge" for most of the miRNA copies. Thus, alongside miRNA copy number, the copy numbers and binding affinities of both the original target of interest and all possible alternative targets are determinants of the extent of miRNA-mediated regulation. RISC component availability, other targeting miRNAs, RNA binding cofactors, and subcellular localization of interaction partners may also affect miRNA-target relations (not shown).

IV. SIV Replication is Directly Downregulated by Four Antiviral miRNAs

SIV Replication is Directly Downregulated by Four Antiviral miRNAs

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Abstract

Background: Host cell microRNAs (miRNAs) have been shown to regulate the expression of both cellular and viral RNAs, in particular impacting both Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV). To investigate the role of miRNAs in regulating replication of the simian immunodeficiency virus (SIV) in macrophage lineage cells, we used primary macrophages to study targeting of SIV RNA by miRNAs. We examined whether specific host miRNAs directly target SIV RNA early in infection and might be induced via Type I interferon pathways.

Results: miRNA target prediction programs identified miRNA binding sites within SIV RNA. Predicted binding sites for miRs-29a, -29b, -9 and -146a were identified in the SIV Nef/U3 and R regions, and all four miRNAs decreased virus production and viral RNA expression in primary macrophages. To determine whether levels of these miRNAs were affected by SIV infection, IFNβ or TNFα treatments, miRNA RT-qPCR assays measured miRNA levels after infection or treatment of macrophages. SIV RNA expression as well as virus production was downregulated by direct targeting of the SIV Nef/U3 and R regions by four miRNAs. miRs-29a, -29b, -9 and -146a were induced in primary macrophages after SIV infection. Each of these miRNAs was regulated by innate immune signaling through TNFα and/or the Type I IFN, IFNβ.

Conclusions: The effects on miRNAs caused by HIV/SIV infection are illustrated by changes in their cellular expression throughout the course of disease, and in different patient populations. Our data demonstrate that levels of primary transcripts and mature miRs-29a, -29b, -9 and -146a are modulated by SIV infection. We show that the SIV 3' UTR contains functional miRNA response elements for all four miRNAs. Notably, these

miRNAs regulate virus production and viral RNA levels in macrophages, the primary cells infected in the CNS that drive inflammation leading to HIV-associated neurocognitive disorders. This report may aid in identification miRNAs that target viral RNAs and HIV/SIV specifically, as well as in identification of miRNAs that may be targets of new therapies to treat HIV.

Keywords: microRNA, miR-29a, miR-29b, miR-9, miR-146a, IFNb, TNFa, HIV, SIV, macrophages

Background

Approximately 34 million people worldwide are currently infected with HIV according to the World Health Organization (http://www.who.int/features/qa/71/en/index.html). As a result of the development of Highly Active Antiretroviral Therapy (HAART), infected individuals are living longer, healthier lives than just two decades ago. Despite this success, patients living with HIV often suffer from complications associated with long-term infection such as cardiac and neurological disorders, in addition to side effects from antiretroviral drugs [1, 2]. Along with HAART treatment, recently there has been research targeted at HIV eradication focused on therapeutic vaccine development [3], purging the latent reservoirs that harbor virus [4], and reducing the expression of the CCR5 co-receptors [5], cell-surface proteins, used by HIV for entry into cells. To this end, a better understanding of HIV-host interactions is needed.

The Type I IFN response is a major host defense against virus infection [6, 7]. HIV infection initiates a cascade of cytokine induction and innate immune signaling. In our SIV macaque model of HIV central nervous system (CNS) disease, analysis of plasma, cerebrospinal fluid, basal ganglia and parietal cortex shows distinct differences in innate immune responses between the periphery and the CNS. This is exhibited by the presence of viral RNA accompanied by induction of IFNβ and IFNβ-inducible MxA in the CNS as early as 4 days after infection [8]. CD4+ T cells are the main cells in the periphery that become infected, in contrast to the central nervous system (CNS), where CD14+ macrophages are the predominant productively infected cells [9-15]. Previous studies have demonstrated that circulating cells from the monocyte/macrophage lineage become infected and traffic to the brain where they induce cytokine signaling and also

infect other macrophage lineage cells and astrocytes [6, 16]. Many cytokines, including IFNβ, are produced by virus-infected or virus-exposed cells. Soluble IFNβ produced by infected cells binds to uninfected cells to signal through the Type I IFN receptor, activating over 100 interferon-stimulated genes as well as antiviral proteins such as MxA [17]. Thus, the signal is amplified and a robust antiviral response is activated to prevent the spread of infection. Cellular restriction factors are another host defense against HIV infection. These include, but are not limited to, TRIM5α [18, 19], APOBEC3G [20, 21], SAMHD1 [22, 23] and microRNAs (miRNAs) [24, 25].

miRNAs are small regulatory molecules that fine-tune levels of target mRNAs in the cell through binding to miRNA response elements (MREs) in target mRNAs. Binding results in post-transcriptional repression caused by target mRNA degradation, translational inhibition and/or sequestration [26]. Each miRNA may have hundreds of predicted target mRNAs, resulting in regulation of expression for close to 60% of human mRNAs [27]. miRNAs regulate human physiology at the level of cell cycle and differentiation [28] and innate immune signaling and antiviral mechanisms [29-32], as well as yet to be identified genes and pathways. Their actions extend into roles in pathogenesis of several viruses including Hepatitis C [30, 33], influenza [34, 35], herpes viruses [36, 37] and HIV.

miRNAs influence several stages of HIV-1 infection. Humans infected with HIV-1 have alterations in miRNA profiles [38] with differences in distinct miRNA populations in patients with varying levels of CD4+ T cell count/plasma HIV RNA copies [39] and elite suppressors [40-43], indicating involvement of miRNAs in response to virus infection. HIV modulates levels of several miRNAs at various time points after infection

[44, 45] and is also regulated by miRNAs in multiple ways [46]. Specific miRNAs target cytokines important in the immune response [47], as well as transcription factors, such as Cyclin T1 [48], which are necessary for transcription of viral genes. Some or all of miRNAs miRs-28, -125b, -150, -223 and -382 are reportedly involved in differential susceptibility of active and resting CD4+ T cells [49, 50] and monocytes and macrophages [51, 52] to HIV infection. Multiple reports suggest direct targeting of HIV-1 by host miRNAs [24, 44, 53-56]. Hariharan et al., used in silico analysis to identify target sites for host miRNAs in HIV-1 *vpr* (miR-149), *vif* (miR-324-5p), *vpu* (miR-378) and *nef*-LTR (miRs-29a and -29b) [53]. A follow-up study by the same group demonstrated miR-29a regulation of Nef expression and HIV-1 replication, and suggested that this was due to miRNA targeting the HIV-1 Nef transcript [24]. The miR-29 family was also reported to inhibit replication of HIV-1, demonstrating that the RNA-induced Silencing Complex (RISC) protein, Ago2, and P body protein, RCK/p54, directly interact with viral RNA in a miR-29a-dependent manner [54]. Nathans, et al. also showed that miR-29a binds the same MRE in HIV-1 as predicted by Hariharan et al [53], and this site is conserved across HIV-1 subsets [54]. Sun et al identified MREs for several miRNAs within HIV-1 and reported downregulation of miRs-21, 155, -29a, -29b and -29c, and an upregulation of miR-223 in response to HIV-1 infection in CD4+ T cells [55]. This group also reported only weak repression of a pNL4-3-Luc reporter by miRs-29a, -29b and -223, hypothesizing that this was due to a hairpin in this region of HIV-1 RNA sequence that interfered with RISC/miRNA binding [55]. In contrast to Sun et al, Schopman et al found induction of miR-29a in several cell types in response to HIV-1 infection [44]. This difference may be due to time after infection when miRNA levels

were measured or the specific cells that were used for the studies. Finally, Houzet et al used anti-HIV-1 miR-326 as an example that sequence complementarity between a miRNA and its target mRNA correlates with inhibited expression of that target mRNA [56]. Together, these reports demonstrate modulation in levels of at least 13 human miRNAs during HIV-1 infection in various cell types. Two of these miRNAs (miRs-29a and -29b) have been validated by more than one study to have an effect on, or to directly target HIV-1 RNA transcripts [24, 53-55].

We have developed a rapid and consistent SIV macaque model of HIV/AIDS and CNS disease in order to study the cellular and viral molecular events and pathogenesis during acute, asymptomatic and AIDS stage of disease [6-9, 57]. We have shown both *in vivo* and *in vitro* that TNFα and IFNβ are induced during acute infection in SIV-infected macaques [7, 8], and both cytokines regulate several miRNAs [30, 32, 58]. We demonstrate here that TNFα and IFNβ induce specific miRNAs at very early time points after SIV infection. SIV infection and cytokine stimulation of primary macrophages were used to dissect the mechanisms of miRNA induction, innate immune signaling and control of virus infection. We evaluated these miRNAs in regard to their effects on virus replication and mRNA levels, ability to target viral RNA sequences and modulation by innate immune signaling pathways. We provide evidence that the four miRNAs, miR-29a, -29b, -9 and -146a, are induced in macrophages during innate immune signaling and target the viral RNA, reducing virus replication and virus production.

Results

Predicted miRNA recognition elements (MREs) in SIV 3' UTR

miRNA target prediction programs [59, 60] were used to identify potential miRNA binding sites within the 3' untranslated region (UTR) of SIV 17E-Fr (Figure 1, Table 1). Many miRNAs were identified that have predicted MREs (miRNA response elements) in the SIV RNA 3' UTR, and we focus here on miRs-29a, -29b, -9 and -146a, (Figure 1A, 1B). All four miRNAs contain promoter binding sites for transcription factors induced during innate immune signaling. miRs-29a and -29b are predicted to contain two ISRE (STAT1/STAT2 heterodimer induced by Type I IFN signaling) GAS (STAT1 homodimer activated by IFNγ signaling) binding sites in the promoter [61] and are induced in response to IFNα/β and IFNγ . The miR-9 promoter contains an NF-κB binding site and is induced by TNFα in an NF-κB-dependent manner [58]. The miR-146a promoter contains binding sites for, and is regulated by, PU.1 and C/EBPα [62], transcription factors induced by innate immune signaling. In addition, the ability of miRs-29a and -29b to target HIV-1 transcripts has been supported by multiple studies [24, 53-55]. The transcriptional activation of these miRNAs, in addition to the predicted binding sites in the SIV RNA sequence, suggests miRs-29a, -29b, -9 and -146a may be induced during the innate immune response and inhibit viral replication.

Effects of miRs-29a, -29b, -9 and -146a on SIV production in primary macrophages

To determine if the miRNAs with predicted binding sites in the UTR of SIV have an effect on virus production, macaque macrophages were transfected or not with each of the miRNAs and infected with SIV twenty-four hours after transfection. Levels of virus

released from cells were measured at 24, 48 and 72 hours post-infection (p.i.). At 24 hours p.i., miR-29a, -29b, -9 and -146a reduced virus production from ~250 pg/ml of p27 protein to below the limit of detection (~60 pg/ml) (Figure 2). There was a statistically significant decrease in virus production at 48 hours post-infection by miRs-29a and -29b. miR-9 continued to reduce p27 levels to below the limit of detection and miR-146a reduced p27 levels to below the limit of detection in cells from one experiment. Virus levels were decreased ~4-fold by miR-29a, ~15-fold by miR-29b and ~8-fold by miR-146a (Figure 2). A statistically significant decrease in virus production was maintained by all four miRNAs through 72 hours post-infection (Figure 2).

miRNA-mediated reduction of full-length and spliced SIV RNA

To investigate whether these miRNAs exert anti-SIV effects through transcript degradation or translation inhibition, total cellular RNA was isolated from the same cells used in Figure 2. Full-length and multiply-spliced (*tat/rev* RNA) viral RNA levels were measured by RT-qPCR. All four miRNAs significantly decreased the levels of full-length (Figure 3A) as well as multiply-spliced (Figure 3B) SIV RNA. Full length and spliced RNA levels were most significantly reduced at 24 hours after infection when viral RNA abundance is lowest (Figure 3A and 3B, left panels). Experiments were also performed using 25 nM of miR-29b and the same reduction in SIV RNA levels was observed using this 4-fold lower miRNA concentration (data not shown).

We hypothesized that miRNA-specific antisense oligonucleotides (antagonists) that decrease the levels of available miRNAs would lead to an increase in viral RNA. Antagonists were transfected into macaque macrophages, and cell lysates were collected

at 48 hours post-infection. We found that miRNA antagonists for miRs-29a, -29b and -146a significantly increased levels of SIV RNA when compared to SIV-infected untreated cells (Figure 3C). Inhibition of miR-9 did not increase viral RNA levels. This was presumably due to the fact that this miRNA is approximately 1000-fold less abundant than miRs-29a and -146a (data not shown), and inhibition by low copy number miRNAs may be inefficient [63, 64].

Effects of miRs-29a, -29b, -9 and -146a on expression of a luciferase reporter

After identifying predicted miRNA binding sites in the SIV 3' UTR, a luciferase assay was performed to examine the effects of the four miRNAs on expression of luciferase from a plasmid containing the predicted sites. Transfection of 293T cells was done with a luciferase reporter plasmid alone, or a plasmid with genomic regions containing the SIV 3' UTR. Luciferase expression was measured in the absence and presence of miRs-29a, -29b, -9 and -146a. Addition of each miRNA resulted in a dose-dependent inhibition of reporter gene expression (Figure 4A). Transfection with two different scrambled mimics had no effect on expression of the reporter gene (Figure 4A).

Direct Targeting of MREs in the SIV 3' UTR

To determine whether the predicted SIV 3' UTR MREs were bound by the predicted targeting miRNAs, we transfected into HeLa cells biotinylated 50-nucleotide RNA oligonucleotide molecules (oligos) that corresponded to wild-type (WT) or mutated (mt) MRE seed-binding SIV U3-R regions (Figure 4B). We calculated the difference in the percent of each miRNA precipitated by WT and mt oligos. Cells were lysed, and the

biotinylated oligonucleotides were selected with strepavidin. Recovered miRNA was quantitated by RT-qPCR. The two predicted SIV 3'UTR seed binding sites for miR-29 family members were included in two oligos corresponding to nucleotides (nts) 10030-10070 and 10063-10112 of the SIV RNA. The one predicted binding site for miR-9 was included in the oligo corresponding to nts 9740-9789 of the SIV RNA. The two predicted sites for miR-146a were included in two oligos corresponding to nts 9680-9729 and 10000-10049 of the SIV RNA (Figure 4B). miRs-29a and -29b bound to the site contained within nts 10063-10112 of the SIV genome, however, neither miR-29a nor miR-29b bound to the predicted site within nts 10039-10070 (Figure 4C). miR-9 bound to its predicted site contained within nts 9740-9789 (Figure 4C). miR-146a bound to the predicted site contained within nts 9680-9729, but did not bind to the predicted site contained within nts 10000-10049 (Figure 4C). These results demonstrate that there are functional binding sites for miRs-29a, -29b, -9 and -146a within the SIV 3' UTR.

Upregulation of mature anti-SIV miRNAs during SIV infection of primary macrophages

To examine whether SIV infection affects the levels of miRs-29a, -29b, -9 and -146a, each of these miRNAs was measured in primary macaque macrophages after SIV infection and compared with miRNA levels in uninfected time-point controls. Levels of all 4 miRNAs increased during infection (Figure 5). Primary macrophages were infected with SIV, and RNA was isolated at 2, 4, 8, 12, 24 and 48 hours post-infection. miR-29a and -29b levels increased together at 12 hours and by 50% and 60%, respectively, at the 48-hour time point (Figure 5A and B). Levels of miR-9 increased approximately 50-85%

4 hours after infection in three out of four animals (Figure 5C). There was also an increase in miR-146a levels at 12 and 24 hours after infection, with the largest average increase being 50% at 24 hours (Figure 5D). These data demonstrate an induction of mature miRNA levels during early stages of SIV infection and suggest that an increase in the levels of these miRNAs may contribute to the decrease in SIV replication and viral RNA levels that we have shown here.

IFNβ-mediated upregulation of precursor and mature anti-SIV mRNAs

The increase in levels of miRs-29a, -29b, -9 and -146 (Figure 5) in response to SIV infection is unlikely to be caused directly by the virus, since the only viral proteins in the cells at this time are from the virion. IFNβ has been shown to modulate expression of miRNAs in our laboratory and by others [30, 32], specifically miRs-29a and -29b [61]. We examined the effects of IFNβ treatment of cells on the expression levels of all four miRNAs. Human macrophages were used so that both the mature and pri-miRNAs could be measured (pri-miRNA assays have not been developed for macaque sequence). Macrophages were treated with IFNβ, and RNA was extracted at 4, 8, 12 and 24 hours after treatment. IFNβ caused a modest but consistent increase in the levels of miRs-29a and -29b at early time points, with no discernable difference observed for miRs-9 and -146a (Figure 6A-D). miR-29a was increased significantly at eight hours by 25% and miR-29b was increased similarly after stimulation with IFNβ (Figure 6A, 6B). Levels of miR-146a showed a similar trend at 8 hours (Figure 6D). This data shows that IFNβ induction early in infection leads to an increase in levels of miR-29a and -29b in primary

human macrophages. IFNβ treatment produces similar results in primary macaque macrophages (Supplemental Figure 1A-D).

The increase in levels of mature miRNAs can be due to a decrease in miRNA turnover, an increase in transcription of precursor miRNAs (pri-miRNAs), or an increase in mature miRNA processing. To address at what level these miRNAs were being increased, pri-miRNA levels were measured in primary human macrophages at 2, 4, 8, 12 and 24 hours after IFNβ treatment (Figure 6E-H). Expression of pri-miRs-29a and -29b was significantly increased ~2-fold at 2, 4 and 8 hours after IFNβ treatment (Figure 6E, 6F). Coordinated regulation of these two miRNAs was expected, as they are part of the same transcript. Expression of miR-146a was increased 44% at 8 hours after treatment (Figure 6H). Transcription of miR-9 was significantly decreased at all time points (Figure 6G), explaining the lack of induction of the mature miRNA by IFNβ. An increase in the pri-miRNA indicates IFNβ stimulation caused an upregulation of the transcriptional activation of the three miRNA.

The canonical response to IFNβ was confirmed by measuring the levels of the interferon-stimulated gene, *mxa*. Both SIV infection and IFNβ stimulation significantly increased *mxa* expression (Supplemental Figure 2A, 2B), and IFNβ also reduced viral RNA levels (Supplemental Figure 2C). These results demonstrate canonical signaling of pathways downstream of IFNβ in our primary macrophage system.

TNFα signaling drives expression of IFNβ and anti-SIV miRNAs

In primary macrophages, IFNβ stimulation modulates levels of pri- and mature miRs-29a and 29b, and pri-miR-146a (Figure 6A-H). Like IFNβ, TNFα is induced during

the acute phase of SIV infection as part of the innate immune response [8], and TNFα has been shown to increase expression of IFNβ and interferon-stimulated genes [65]. TNFα stimulation of primary macrophages resulted in an increase in the IFNβ-stimulated gene, *mxa* (Figure S3), demonstrating regulation of this pathway by TNFα. To test the effect of TNFα stimulation on miRNA levels, primary macrophages were treated or not with TNFα and cells harvested at 2, 4, 8, 12 and 24 hours after treatment. TNFα stimulation resulted in an increase in miR-29a and -29b levels by an average of 30% and 35%, respectively, at 12 hours (Figure 7A, B). miR-9 levels increased significantly at 2 and 24 hours after treatment by 40% and 90%, respectively (Figure 7C). Levels of miR-146a increased maximally by 55% at 24 hours (Figure 7D). Macaque macrophages responded similarly to stimulation with TNFα (Figure S4A-D).

TNFα stimulation also induced transcriptional expression of all four miRNAs (Figure 8E-H). pri-miR-29a expression increased by ~45% 2 hours after treatment, with a maximum increase of 57% 4 hours after treatment (Figure 7E). Similarly, pri-miR-29b expression increased maximally 2 hours after treatment by 55% and remained elevated by ~40% 4 hours (Figure 7F). There was a dramatic 10-fold increase of pri-miR-9 2 hours after treatment with TNFα. This increase was maintained at 5-fold 4 hours, and 2-fold through the 12 hour time point (Figure 7G). pri-miR-146a expression increased by 2-fold 2 and 4 hours after treatment. An approximate 50% increase was maintained through the 12-hour time point (Figure 7H).

Discussion

The identification of miRNAs as regulators of gene expression has dramatically changed the understanding of post-transcriptional regulation of cellular and viral genes. A number of viruses encode miRNAs in their genome that regulated cellular genes required for replication [66]. We have previously shown that miRNAs in plasma of SIV-infected macaques provide a signature of infection and progression to CNS disease, demonstrating that SIV infection affects miRNA expression *in vivo* [67]. In this study, we demonstrate for the first time that four miRNAs directly bind to the U3 region of the SIV RNA. Further, all four miRNAs, miR-29a, -29b, -9 and -146a, controlled SIV virus production and replication by decreasing SIV full length and spliced *tat/rev* RNAs in infected primary macrophages. In addition we provide evidence that these four miRNAs are transcriptionally regulated by the innate immune response, specifically, TNFα and Type I IFN. We have shown previously that virus replication in macrophages increases both of these cytokines and thus, it appears that these miRNAs are part of the innate antiviral immune response to SIV.

The protein products of spliced *rev* and *tat* transcripts are critical early in infection for progression to later stage and productive infection. The 3' UTR of SIV contains MREs for all four miRNAs, miRs-29a, -29b, -9 and -146a. All SIV RNAs share the same 3' UTR sequence and therefore, these four miRNAs have the capability to target all full-length and spliced viral transcripts for degradation. In order to have a significant impact on virus infection and the propagation of virus, one would hypothesize that miRNAs would target the early multiply-spliced genes, preventing the progression of virus replication to the productive stage. In addition, we hypothesized that the levels of

the miRNAs would be high in the cell or would increase within hours following infection in a cell or be upregulated in response to early cytokines in bystander cells, preventing virus spread to other cells. Levels of the anti-SIV miRNAs studied here increased as early as 4 hours post-infection, and miRs-29a, -29b and -146a were significantly induced by 24 and 48 hours after infection, respectively. From these results, we see that viral infection in macrophages upregulates miRNAs that regulate the virus.

It has been shown for virus infections, including HIV and SIV, that infection of cells, particularly macrophages, leads to the induction of innate immune responses, Type I IFN and TNFα [8, 17]. To determine whether the increased levels of the four miRNAs that reduce virus infection were induced by the cellular response to infection, we used INFβ and TNFα to examine the induction of mature as well as pri-miRNAs. INFβ and/or TNFα, two cytokines that are made in response to SIV infection in macrophages increased the four miRNAs. INFβ increased miRs-29a and -29b as early as 2 hours after treatment. TNFα, in contrast increased levels of miRs-29a, -29b and -146a at 12 hours suggesting that its effect was due to stimulation of Type I IFN. TNFα may have a direct effect on miR-9 since the miRNA was significantly increased at 2 hours and appears not to be modulated through the Type I IFN response. Overall, levels of the mature form of these miRNAs increased approximately 50% over control.

While these increases were modest, we found them consistently, and it is important to remember that viral infection drastically alters the pre-existing miRNA-target network in many ways. Upon infection, host miRNAs that may have multiple host targets are provided with many copies of a new, viral target. The abundance of cellular transcript targets may also be modulated. Since the number of targets of a specific

miRNA and the affinity of the targeting miRNA for each target together influence the magnitude of regulation along with the abundance of the miRNA itself, large fold changes or indeed any modulation of targeting miRNA are not necessarily prerequisites for regulation of an evolutionarily novel target. Further investigations of the entire network are needed to establish the necessary stoichiometries for effective regulation. We would also note that this miRNA/target network is even more complicated *in vivo*, as levels of the characterized miRNAs may be altered in cell types other than macrophages. These miRNAs may traffic between cells [68] and may be recycled within a given cell [69], thereby enhancing their effects.

A second important finding is that innate immune responses induced these miRNAs at the transcriptional level. During this early time after infection, SIV spliced RNA encoding Rev and Tat are produced, and this is likely the time when miRNA levels are most efficiently targeting viral transcripts to reduce virus replication. Expression of each miRNA transcript is controlled by specific transcription factors. The promoters of miRs-29a, -29b, -9 and -146a all contain one or more binding sites for NF-κB [58, 70, 71], a transcription factor induced by innate immune signaling. These reports show that miRs-9 and -146a are induced by TNFα and induction of miR-146a is dependent on the three NF-κB binding sites found in its promoter. In cancer cells, TLR signaling and NF-κB activation were shown to suppress expression of miRs-29a and -29b [70], but the promoter for these two miRNAs contains binding sites for several other transcription factors related to innate immune signaling.

While miRs-29a, -29b, -9 and -146a did not evolve to target SIV, they reduced virus production via direct interaction with specific sequences in the 3' UTR of SIV

RNA. In addition, these and other miRNAs may modulate virus infection directly as well as indirectly. For example, miR-29a has been shown to target IFNγ [72]. A report by Chiang et al, demonstrates direct binding of Cyclin T1 by miR-29b and [73], a transcription factor necessary for replication of HIV-1. miR-9 is induced by TLR signaling and NF-κB activation and regulates expression of the NF-κB p50 subunit [58, 74]. miRNA-146a is a negative regulator of innate immunity and overexpression results in downregulation of Type I IFN in PBMCs due to targets within IRAK1 and TRAF6 [71, 75-78]. miR-146a was also reported to target and inhibit expression of CXCR4 [79], a cell surface receptor used by HIV-1 and certain strains of SIV. miRNAs are differentially expressed at various times in different tissues. Furthermore, individual miRNAs may have different factors controlling maturation as well as rates of degradation. More investigation is needed to elucidate the connections between IFNβ/TNFα signaling and our observed modulation of transcription of these miRNAs. What is becoming clear is that this miRNA response reflects another downstream antiviral innate immune effector.

Several lines of evidence suggest that miRNAs may be useful as therapeutic inhibitors of HIV-1 infection. These data include: 1) a relationship between differences in miRNA profiles and cell type susceptibility to HIV-1 infection [49-52]; 2) dysregulation of miRNAs during HIV-1 infection [39] and 3) direct miRNA targeting of HIV-1 RNA sequences [54-56]. These reports show the potential for miRNAs to be given therapeutically as potent inhibitors of HIV-1 infection. The main obstacle is delivery of these molecules to a desired tissue or cell population. Several groups have reported nanoparticle delivery of antiretrovirals and siRNAs in HIV-infected cells *in vitro* as well

as in mouse models of neuroAIDS [80]. These methods appear promising for delivery to specific cell and tissue types, as well as only to cells that are infected. Obad et al demonstrated using short LNA antagomiRs to target entire miRNA families [81], and this strategy could be used to inhibit miRNAs that are overexpressed during infection and increase either virus infection or contribute to pathogenesis. We show here that miRs-29a, -29b, -9 and -146a are four anti-SIV miRNAs that have the potential to be used as therapeutic agents against SIV infection.

Conclusion

To our knowledge, this study is the first report linking viral as well as innate immune modulation of miRNAs with direct targeting of viral RNAs and inhibition of virus production. The significant role of miRNAs in the pathogenesis of HIV is underscored by the abundance of reports demonstrating miRNA effects on disease progression as well as effects of virus on miRNA expression. The early induction of miRs-29a, -29b, -9 and -146a caused by SIV and the significant inhibition of virus production very early in infection suggests potential for using these as well as other miRNAs for treatment of HIV in addition to other infectious diseases. Our macaque model of SIV provides an ideal model for testing delivery and efficacy of treatment with miRNAs. Future strategies include using anti-SIV miRNAs or miRNAs that target transcription factors and other proteins necessary for replication of HIV and SIV. Targeted delivery to infected cells or individual cell types may reduce negative off-target effects suffered by many patients currently on HAART.

Methods

Ethics Statement

Animal studies were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (Protocol # PR09M296) and conducted in accordance with the Weatherall Report, the Guide for the Care and Use of Laboratory Animals, and the USDA Animal Welfare Act.

miRNA Prediction Programs

The SIV 3' UTR sequence, consisting of *nef/U3-R* (SIV/17E-Fr nucleotides 9462-10155), was analyzed for miRNA binding sites. Results from miRanda [59] and RNAhybrid [60] were compiled to identify predicted miRNA binding sites within the SIV 3' UTR. The minimum free energy cutoff for miRNA consideration was set at -20 kcal/mol, and the free energy of each miRNA/target site interaction is in supplemental Table 1.

Luciferase Assays

The SIV 3' UTR (*nef/U3-R*, SIV 17E-Fr nts 9458-10160) was cloned into the psiCHECK-2 vector (Promega) 3' of a Renilla luciferase reporter gene. 293T cells were plated at 80,000 per well of a 24-well plate. Cells were approximately 90% confluent 24 hours later for co-transfection of individual miRNA mimics and either psiCHECK-2 plasmid plus SIV insert or psiCHECK-2 plasmid with no insert. Controls were transfected with either psiCHECK-2 or psi-CHECK-SIV 3' UTR, but no miRNA mimics. Cells were harvested 24 hours later and assayed for luciferase expression using the Renilla Luciferase Assay System (Promega) and measured on (Fluoroskan Ascent FL,

Thermo Scientific). The raw luciferase value of each sample was normalized to total protein for that sample to control for well-to-well differences in cell number. Firefly luciferase was not used for normalization as all four miRNAs reduced levels of this control reporter. Several individual plasmids were co-transfected to use as transfection controls but were not able to be included because expression of all was modulated by some or all of miRs-29a, -29b, -9 and -146a. Experiments were repeated replacing the miRNA mimics with scrambled mimics (scrambled #1 Ambion mirVana miRNA mimic Negative Control #1, scrambled #2 Sigma Mission miRNA Negative Control 2).

Macrophages

Isolation and culture

Macaque macrophages were obtained from rhesus and pigtail macaque donors. Human macrophages were obtained from leukopacks from anonymous donors of the Johns Hopkins Hospital HATS donation center. Whole blood was diluted with HBSS, loaded onto a Ficoll gradient and centrifuged for 30 minutes at 2000 rpm. Plasma was removed and PBMCs were removed from the interface of plasma and Ficoll. Cells were washed in HBSS and pelleted (1500 rpm for 10 minutes) twice prior to red blood cell lysis (15 ml of 155 mM NH_4Cl, 10 mM $KHCO_3$ and 1 mM EDTA for 15 minutes at 37°C). 35 ml of HBSS was added, and PBMCs were centrifuged to remove red blood cell lysis buffer. Cells from individual donors were plated separately (donor samples were not pooled) at $4x10^6$ cells per well in 12-well plates or $10x10^6$ cells per well in 6-well plates and cultured for 7 days in medium containing M-CSF and 20% autologous serum. One half of the total volume of medium was replaced on day 3. On day 7, cells were washed 3 times

with PBS to remove non-adherent cells. The medium was replaced the same as above, only with 10% serum. Infection and treatments were all performed on day 8 after plating.

miRNA Transfection and SIV Infection

Rhesus or pigtail macaque macrophages were approximately 90% confluent on day eight post-plating when transfected with 100 nM of individual miRNAs or miRNA antagonists (Qiagen) for 6 hours using Lipofectamine 2000 (Invitrogen/Life Technologies) diluted in OptiMEM Reduced Serum Medium (Life Technologies) as per manufacturer's instructions. Mock transfections included Lipofectamine 2000 and OptiMEM, but no miRNA mimic.. Twenty-four hours later, cells were infected with macrophage tropic SIV 17E-Fr [GenBank:AY033146.1] at an MOI of 0.05 for six hours. Following infection, cells were washed 3 times with PBS, and fresh medium was added. Supernatants and cells were collected at 24, 48 and 72 hours post infection to measure virus production and viral RNA levels, respectively. All results shown are the average of at least three separate experiments.

IFNβ and TNFα Treatment

Day 8 pigtail macaque macrophages were approximately 90% confluent when treated with either 100 U/ml IFNβ (PBL Interferon Source) or 20 ng/ml TNFα (R&D Systems, human 210-TA-010, macaque 1070-RM-025). Total RNA was isolated from control and treated samples at 2, 4, 8, 12 and 24 hours post-infection. All results shown are the average of at least three separate experiments.

miRNA Isolation

All RNA isolation was performed using the mirVana miRNA Isolation Kit (Ambion). Cells were harvested in 600 µl Lysis/Binding Buffer and eluted with 100 µl water. Samples were treated with 2 µl TURBO DNase (Ambion) for 45 minutes at 37°C. Samples were re-extracted using the mirVana miRNA Isolation Kit cleanup protocol and eluted with 100 µl water.

Analysis of SIV p27

200 µl of supernatant was used for p27 assays (Zeptometrix). The assay was performed following an overnight incubation of samples in lysis buffer at 37°C. p27 levels were determined based on the manufacturer's provided standard. All results shown are the average of at least three separate experiments.

RT-qPCR

Results are reported as fold change using the ΔΔCq method. All statistics reported represent 2-tailed t tests assuming unequal variance, performed on ΔΔCq values of time-point matched samples with untreated/uninfected controls. All results shown are the average of at least three separate experiments.

SIV Transcripts

250 ng of RNA was used for reverse transcription cDNA synthesis (Superscript III, Invitrogen/Life Technologies). RT-qPCR was performed as described previously using primers and probes specific for SIV *gag* and *tat/rev* transcripts [82]. Quantification cycle

(Cq) values were normalized to an average of the GAPDH and18S Cq values as well as to time-point controls.

Taqman RT-qPCR – mature miRNAs

10 ng of total RNA was used for reverse transcription cDNA synthesis using the Taqman microRNA Reverse Transcription Kit (Applied Biosystems) and Taqman assays for individual miRNAs. 5 μl of cDNA was used for RT-qPCR with individual miRNA Taqman assays. Cq values were normalized to a U6 internal control as well as to time-point controls.

Taqman RT-qPCR – pri-miRNAs

100-250 ng of total RNA was used for reverse transcription cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). 4 μl of cDNA was used for RT-qPCR with Taqman precursor miRNA assays for individual pri-miRNAs. Cq values were normalized to a U6 internal control as well as to time-point controls.

RNA Immunoprecipitation

HeLa cells were plated using 200,000 cells per well of a 6-well plate and 24 hours later were approximately 90% confluent when transfected with Lipofectamine 2000 (Invitrogen) with 400 pmol of a biotinylated 50-nucleotide WT or mutant RNA oligo corresponding to the predicted regions of binding for individual miRNAs (see Figure 5A). Twenty-four hours after transfection cells were fixed in 0.5% formaldehyde for 15

minutes at room temperature. Cross-linking was stopped by 5 minute incubation at room temperature in 0.25 M glycine, pH 7. Fixed cells were lysed in modified RIPA buffer [82] for 30 minutes at 4°C. Lysed cells were centrifuged for 10 minutes at 14,000 rpm at 4°C. 5% of total sample volume was taken for input. Lysates were incubated for 30 minutes at room temperature with 100 µl of streptavidin beads (Dynabeads MyOne Streptavidin C1, Life Technologies) to pull down endogenous miRNAs bound to the biotinylated oligos. Beads were washed 3 times with 500 µl RIPA Buffer. After reverse cross-linking (5 minutes at 65°C in 100µl of 95% formamide, 10 mM EDTA pH 8.0), 500 µl of mirVana Binding/Lysis Buffer was added to the supernatant and RNA was extracted using the mirVana miRNA Isolation Kit (Ambion). 10 µl of total RNA was used in Taqman miRNA-specific qPCR primer/probe assays to detect specific miRNAs bound to individual oligos containing predicted miRNA binding sites. Percent of pull down by each oligo was determined and normalized by the Percent Input Method (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/epigenetics-noncoding-rna-research/Chromatin-Remodeling/Chromatin-Immunoprecipitation-ChIP/chip-analysis.html).

Statistical Analyses

For mRNA and miRNA comparisons that only consisted of two groups (uninfected/untreated versus infected/treated) a paired, two-sided t test was performed on the well-specific observations (relative to the control average) at each time point. Each miRNA was statistically evaluated as the duplicate- or triplicate-averaged difference from C(q) U6 or 18S and GAPDH relative to control. Statistical significance, p-value <0.05, indicates an effect of intervention. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

JMS, KWW and JEC conceived of and managed the study. JEC provided resources. JMS performed the experiments. JMS and KWW analyzed the data and wrote the manuscript. PMT analyzed data and guided statistical analyses for all experiments. KWW and JEC contributed to revision of the manuscript. All authors read drafts and approved the final manuscript.

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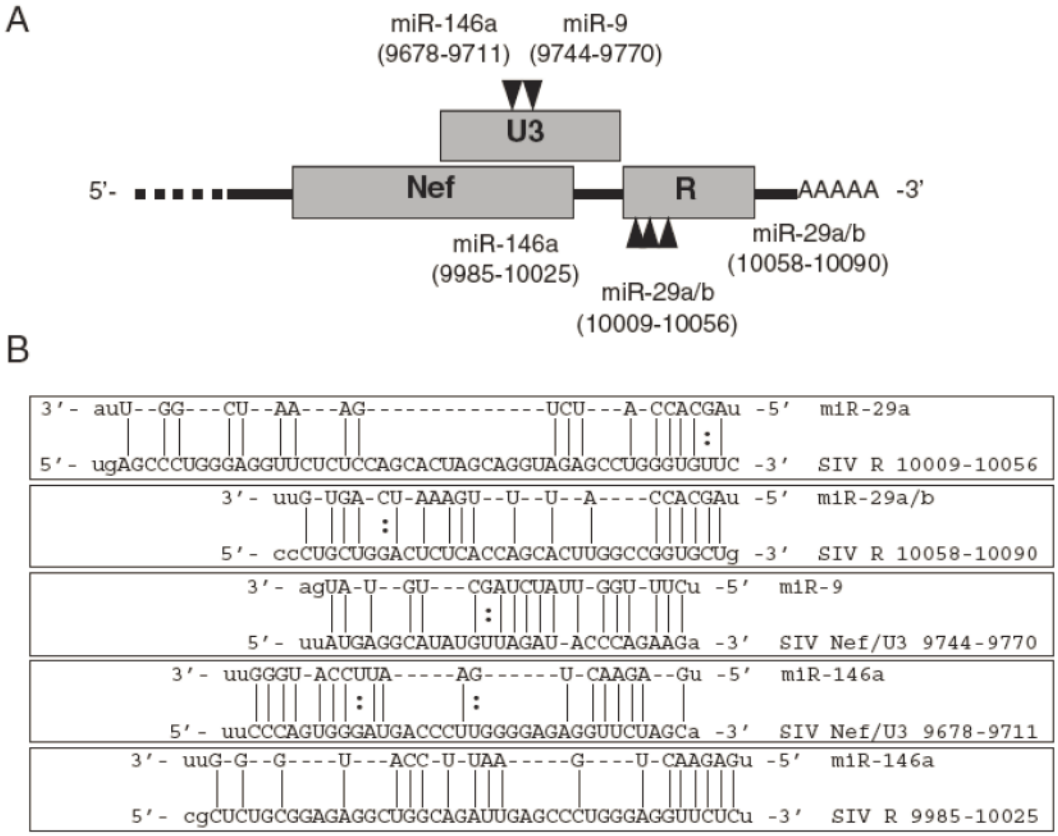

Figure 1. Predicted miRNA binding sites within the 3' UTR of SIV. miRanda and RNAhybrid prediction programs identified miRNA response elements (MREs) for miRs-29a, -29b, -9 and -146a. A) A graphic representation of the SIV 3' UTR with predicted MREs. B) Alignment of MREs within the SIV 3' UTR generated from predictions.

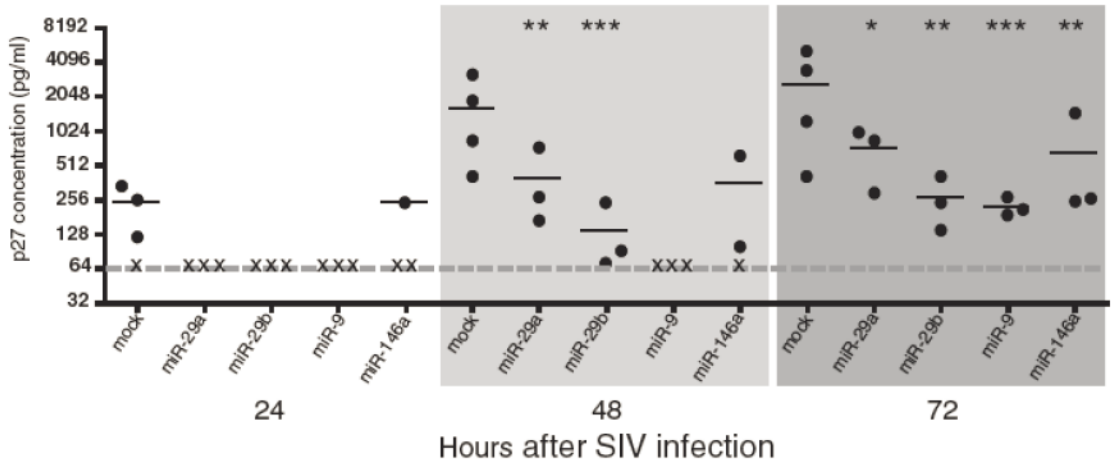

Figure 2. Four miRNAs reduce levels of SIV virus production. Primary macaque macrophages were transfected with 100 nM miRNA mimics, then infected with SIV (MOI 0.05) 24 hours after transfection. Supernatants were collected at 24, 48 and 72 hours post-infection and analyzed for virus production. Data shown is an average of at least 3 experiments for each miRNA at each time point. (x) indicates levels at or below the limit of detection, ~60 pg/ml (gray line). Results reported are p27 pg/ml values averaged from at least 3 separate experiments. All statistics reported are the result of a paired, two-sided t test performed on p27 values of time-point matched samples with untransfected controls. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

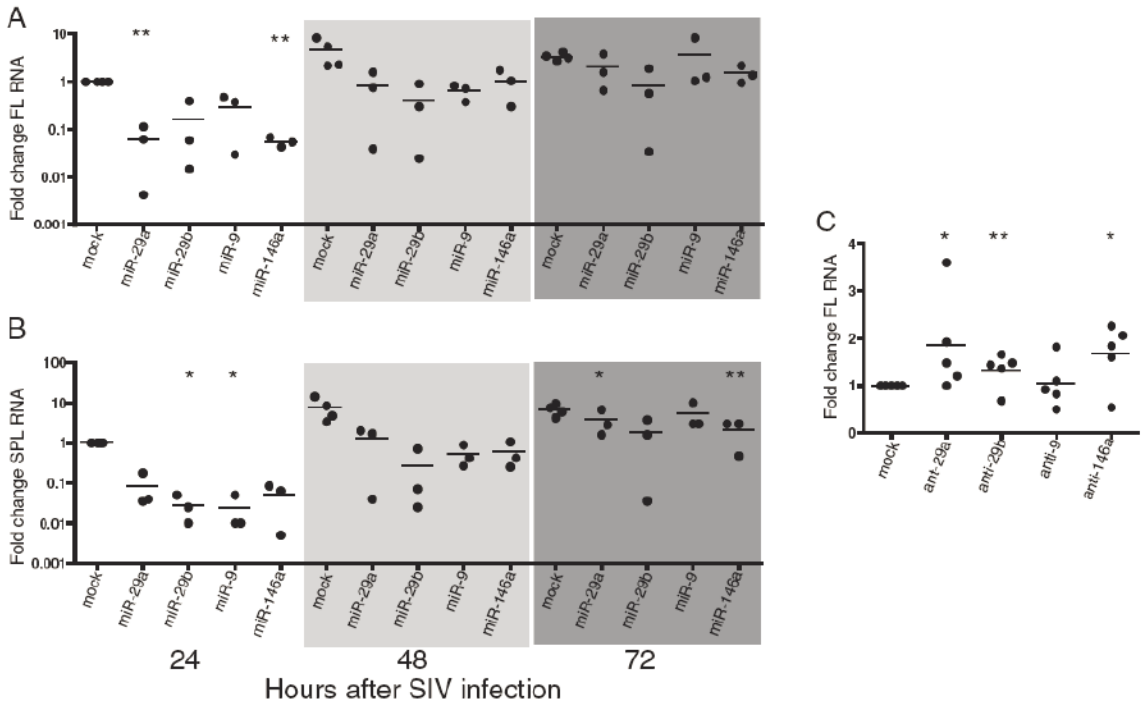

Figure 3. Four miRNAs reduce levels of full length and multiply spliced viral RNAs. Primary macaque macrophages were transfected with 100nM of either miRNA mimics or antagonists, then infected with SIV (MOI 0.05) 24 hours after transfection. Cell lysates were collected at 24, 48 and 72 hours post-infection for miRNA experiments and 48 hours for miRNA antagonist experiments. Levels of full length (A, C) and spliced (B) RNA was measured by RT-qPCR. Values are expressed as fold change over SIV-only controls. Results are reported as percent of SIV-only control using the ΔΔCq method.

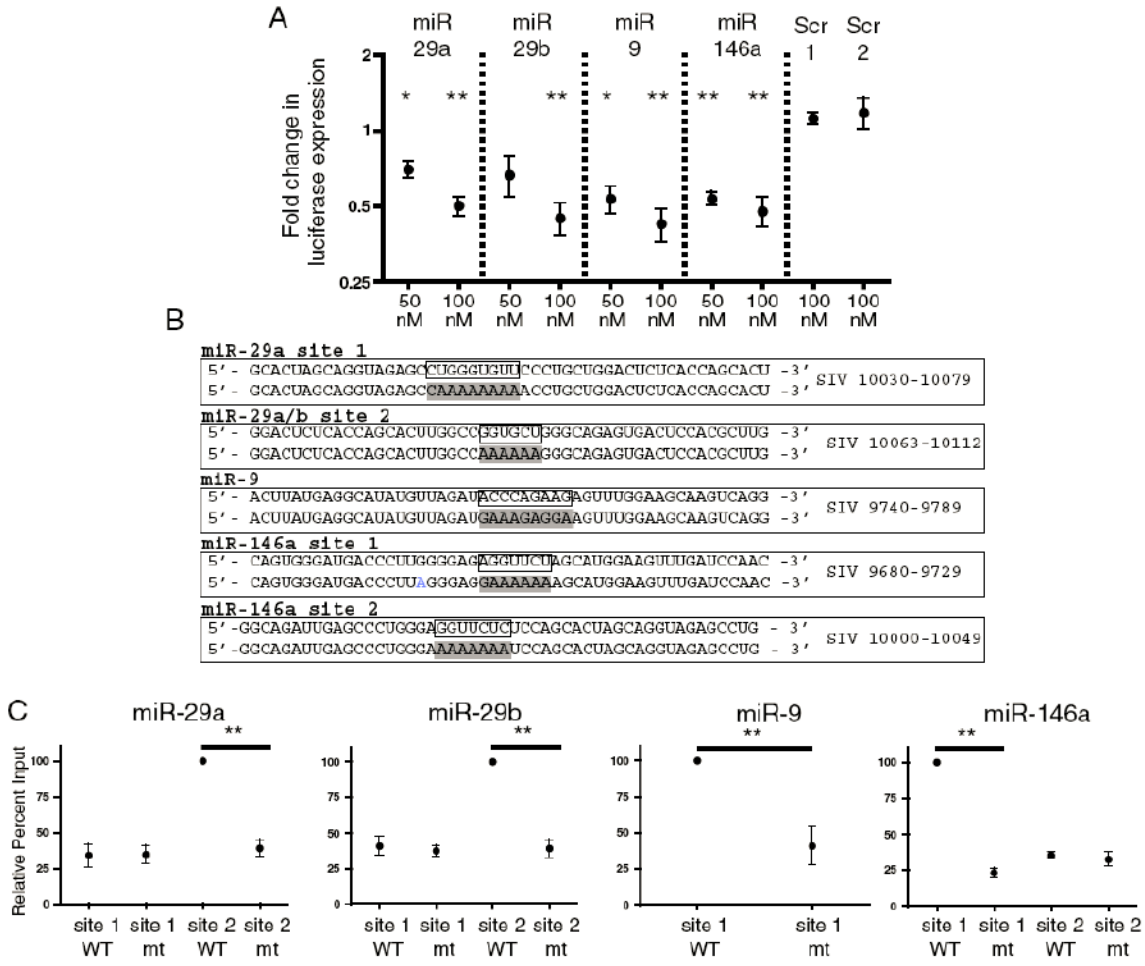

Figure 4. miRs-29a, -29b, -9 and -146a reduce expression of a luciferase reporter and target predicted binding sites in the 3' UTR of SIV. A) SIV *nef/U3-R* region was cloned into the psiCHECK-2 plasmid (Promega). 293T cells were co-transfection with 50 nM or 100 nM of each individual miRNA mimic or scrambled mimic and 100 ng of either psiCHECK-2 + SIV insert or psiCHECK-2 alone. Control samples were transfected with either psiCHECK-2 or psi-CHECK-SIV 3' UTR, and no miRNA mimics. Cells were harvested 24 hours after transfection. Data shown is an average of four separate experiments and represents miRNA effect on luciferase expression for psiCHECK-SIV 3' UTR over psiCHECK only. B) WT and mutant biotin-labeled oligos corresponding to SIV RNA sequences containing predicted miRNA binding sites. WT oligo is show on top and mutant on the bottom. Boxed bases on the WT oligo denote miRNA seed binding site in SIV sequence. Mutant oligo highlighted bases denote the change in the seed-binding region. Annotation is based on SIV sequence AY033146. C) 100 pmol of individual WT or mt oligos were transfected into HeLa cells and lysates collected 24 hours after transfection. Oligos were pulled down using Streptavidin beads and assayed for binding to endogenous cellular miRNAs using Taqman RT-qPCR. Percent of input was calculated for each sample (see methods) and data is presented as (% of input mt)/(% of input WT) with % of input of WT set to 100%. In each experiment, the value of the oligo which pulled down the highest percentage of the

individual endogenous miRNA was set to 100% and binding by other oligos was compared to this value. Data shown is an average of 4 experiments. Statistics represent the comparison of percent of miRNA bound to the WT oligo compared to percent of miRNA pulled down by the corresponding mt oligo and are reported as a two-tailed t test assuming unequal variance.

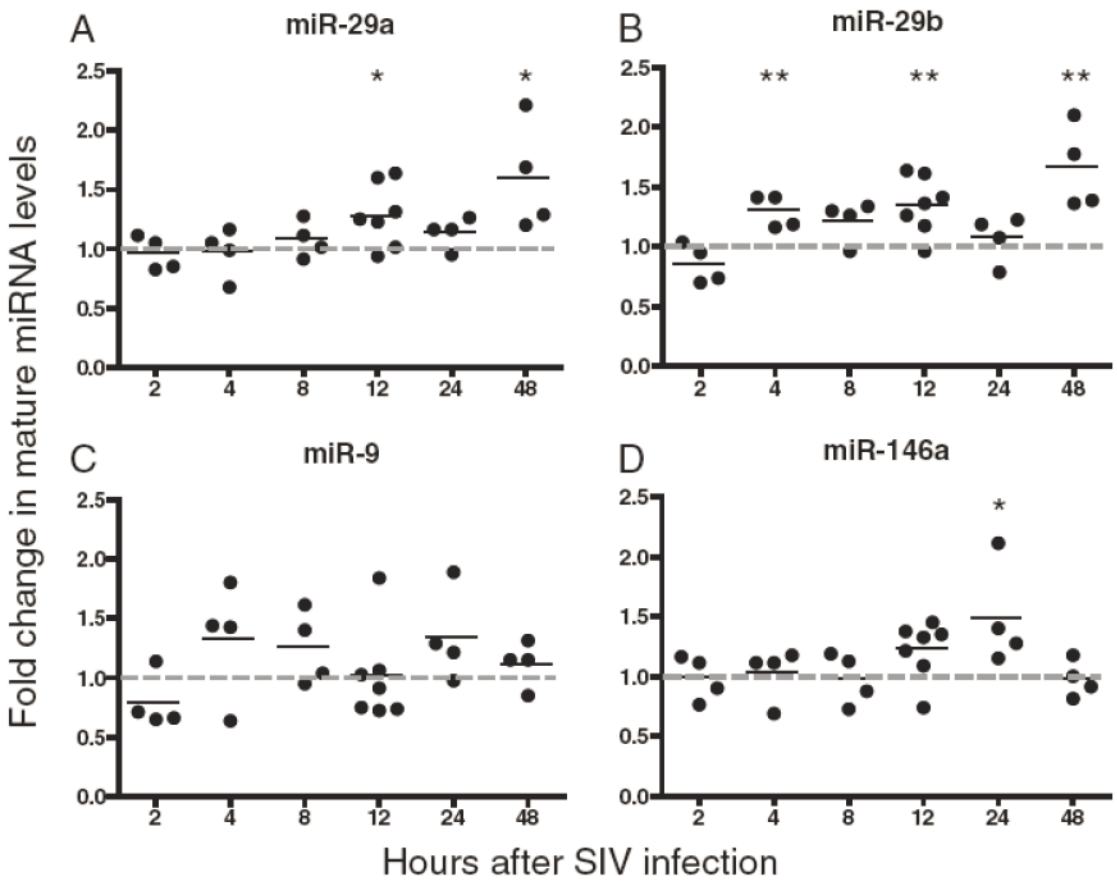

Figure 5. SIV infection increases levels of mature miR- 29a (A), -29b (B), -9 (C) and -146a (D) . Macaque macrophages were infected with SIV (MOI 0.05). Cells were harvested at 2, 4, 8, 12, 24 and 48 hours after infection and RNA was isolated. Taqman miRNA RT-qPCR assays were used to measure levels of mature miRNAs. Results were normalized to U6, then to uninfected samples for the individual time points (ΔΔCq method). Values are expressed as fold induction of miRNAs over uninfected controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

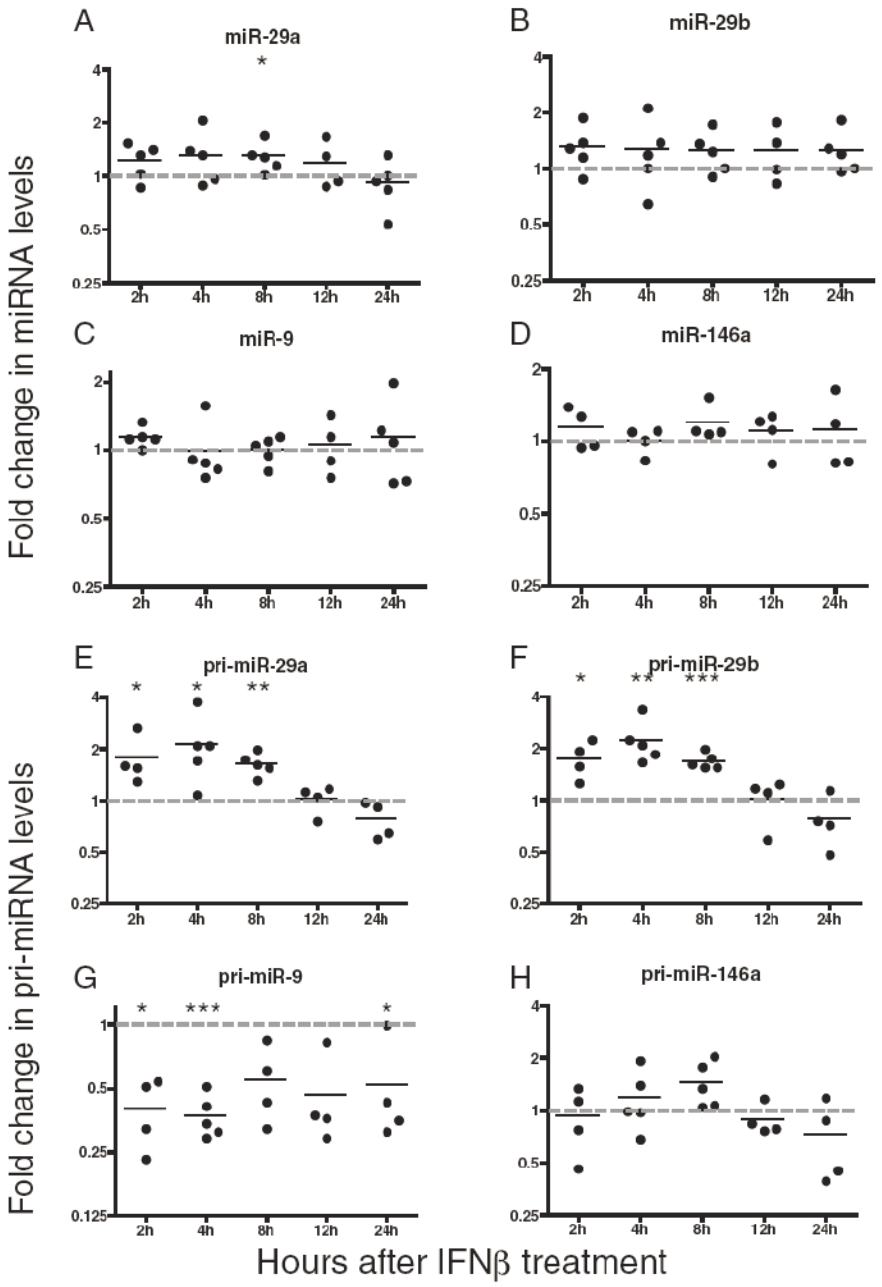

Figure 6. IFNβ increases levels of mature miRNAs and primary miRNA transcripts in human macrophages. Human macrophages were treated with 100 U/ml IFNβ and cells were harvested at 4, 8, 12 and 24 hours after treatment and RNA isolated. A-D) Taqman miRNA RT-qPCR assays were used to measure levels of mature miRNAs. E-H) Taqman pri-miRNA RT-qPCR assays were used to measure levels of primary miRNA transcripts. Results were normalized to U6. Values are expressed as fold induction of miRNAs over uninfected controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

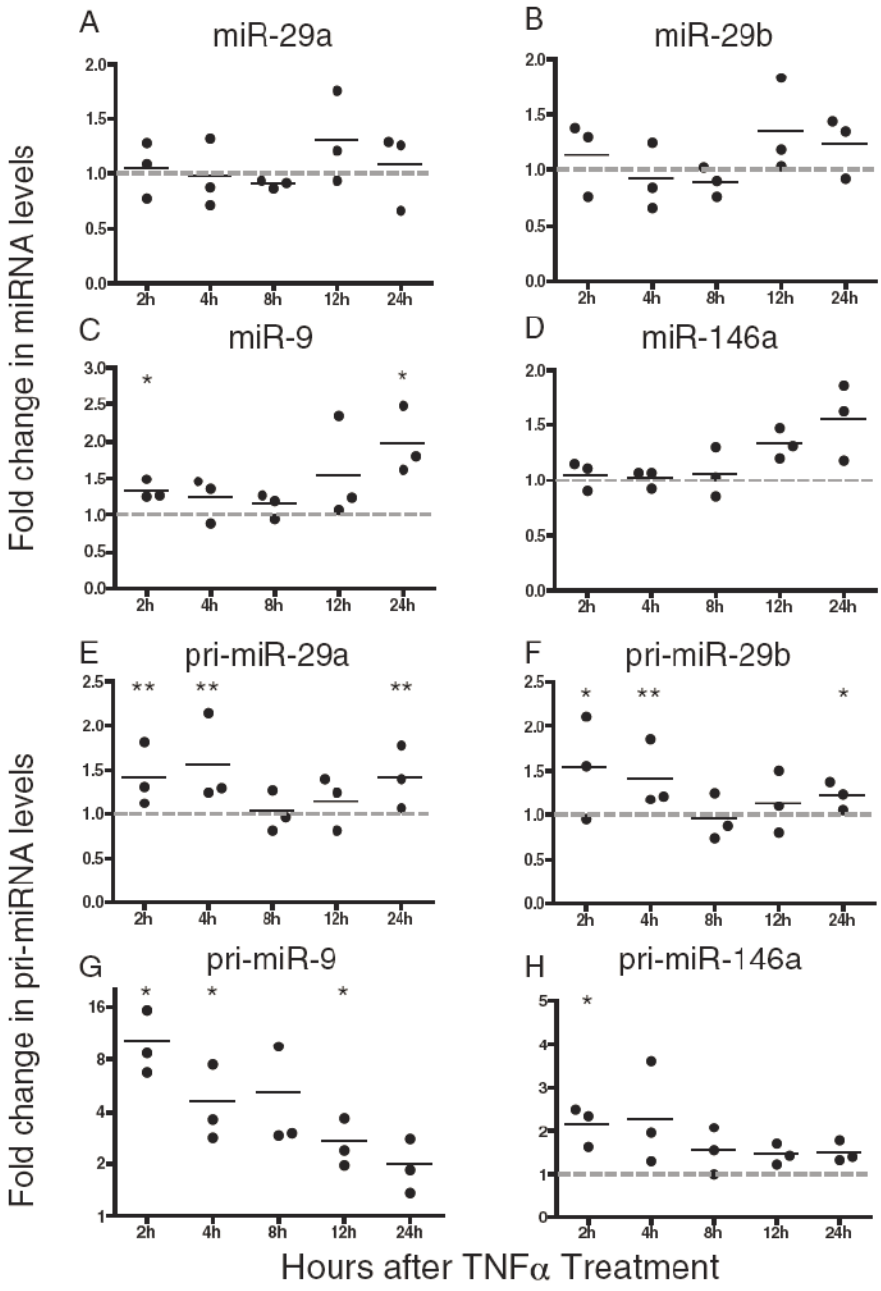

Figure 7. TNFα increases levels of mature miRNAs and primary miRNA transcripts in human macrophages. Human macrophages were treated with 20 ng/ml TNFα. Cells were harvested at 2, 4, 8, 12 and 24 hours after treatment and RNA isolated. A-D) Taqman miRNA RT-qPCR assays were used to measure levels of mature miRNAs. E-H) Taqman pri-miRNA RT-qPCR assays were used to measure levels of primary miRNA transcripts. Results were normalized to U6. Values are expressed as fold induction of miRNAs over uninfected controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

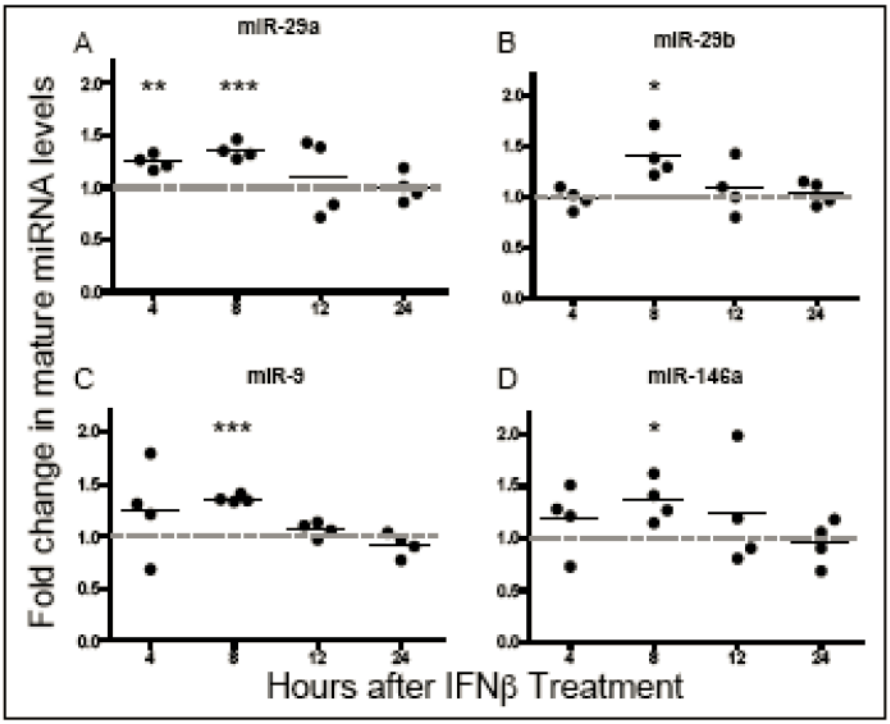

Figure S1 A-D. IFNβ increases levels of mature miRNAs in macaque macrophages. Macaque macrophages were treated with 100U/ml IFNβ. Cells were harvested at 4, 8, 12 and 24 hours after treatment and RNA was isolated. Taqman miRNA RT-qPCR assays were used to measure levels of mature miRNAs. Results were normalized to U6. Values are expressed as fold induction of miRNAs over untreated controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

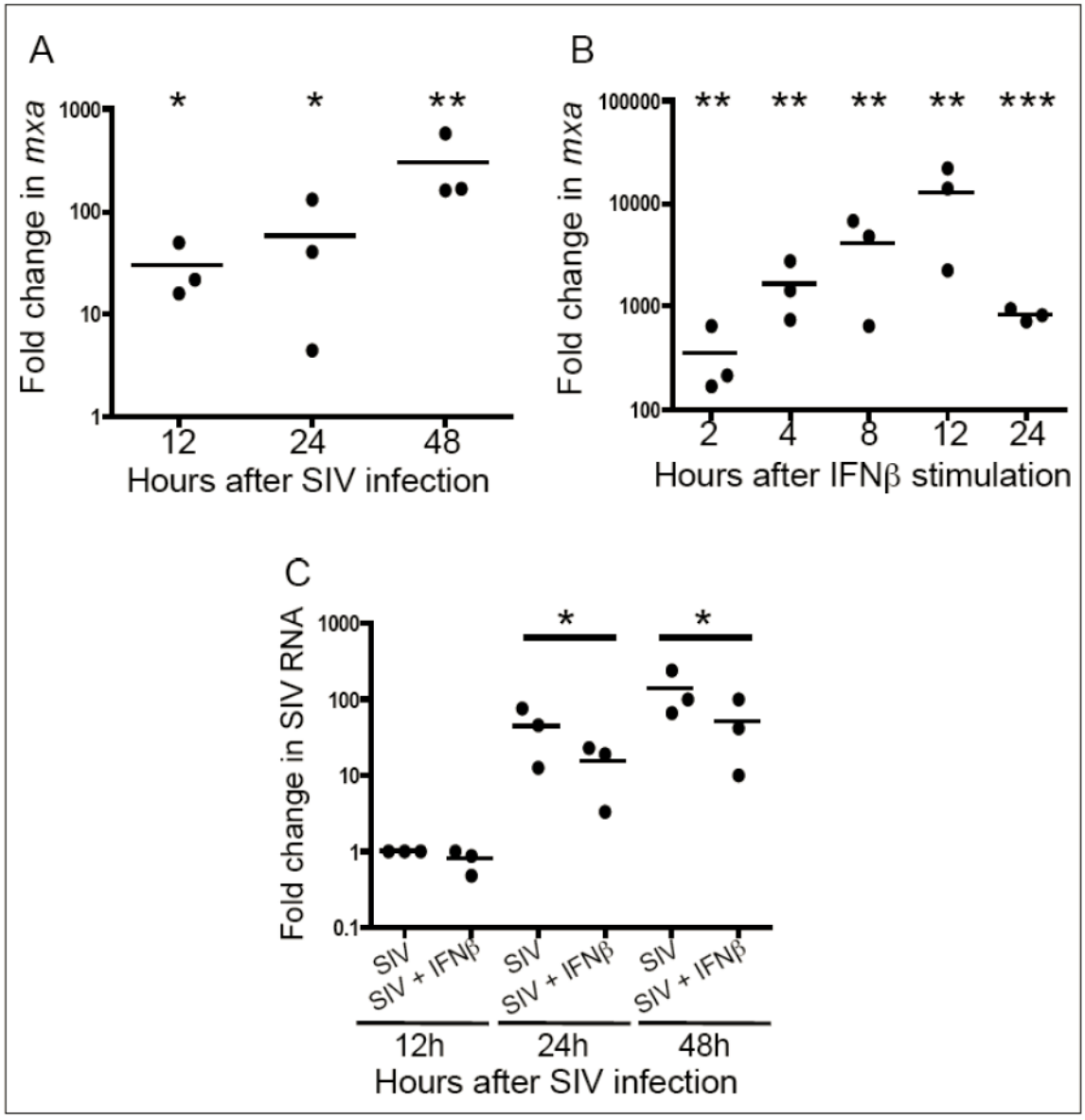

Figure S2. SIV infection and IFNβ stimulation increases *mxa* levels. IFNβ decreases SIV RNA levels. Macaque macrophages were infected with SIV (A), infected with SIV and treated with IFNβ (C) and primary human macrophages were treated with IFNβ (B). RT-qPCR using sequence-specific primers and probe for *mxa* was used to measure *mxa* and SIV RNA levels. Results were normalized to 18S. Values are expressed as fold induction of *mxa* over uninfected/untreated controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

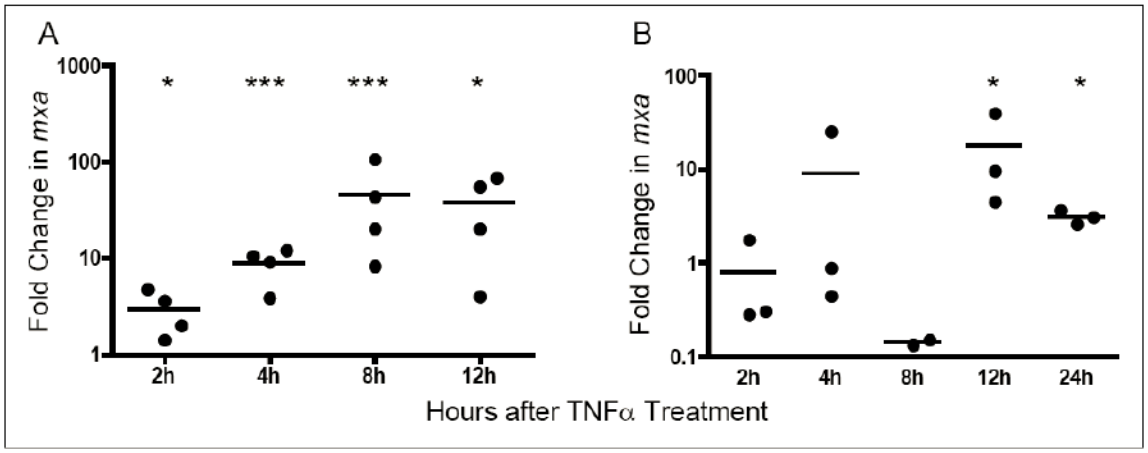

Figure S3. TNFα induced expression of *mxa* in primary macaque and human macrophages. Macaque (A) and human (B) macrophages were treated with 20 ng/ml macaque or human TNFα. RNA was isolated at 2, 4, 8, 12 and 24 hours after treatment. Sequence-specific primers and probe for *mxa* were used for RT-qPCR. Results were normalized to 18S. Values are expressed as fold induction of *mxa* over untreated controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

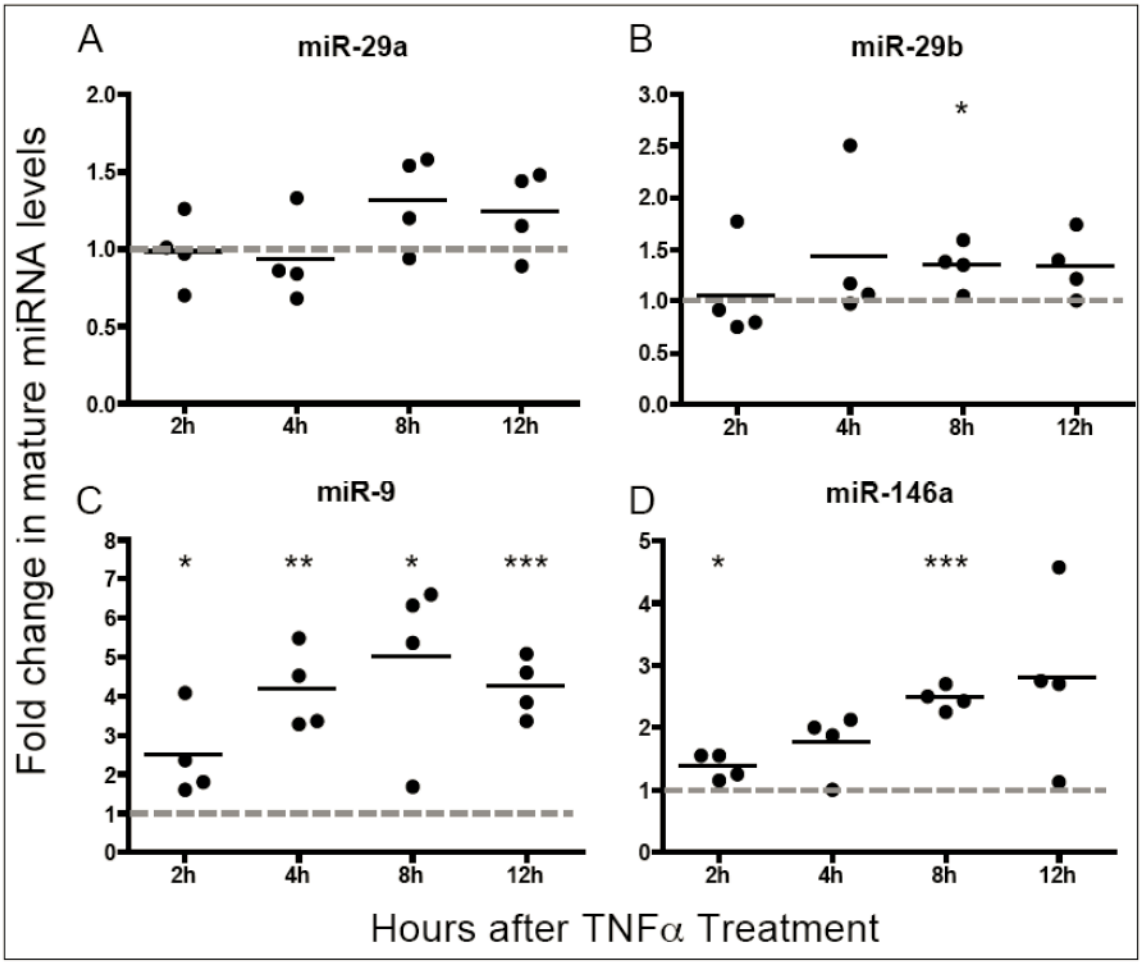

Figure S4A-D. TNFα increased levels of mature miRNAs in macaque macrophages. Macaque macrophages were treated with 20 ng/ml TNFα. Cells were harvested at 4, 8, 12 and 24 hours after treatment and RNA isolated. Taqman miRNA RT-qPCR assays were used to measure levels of mature miRNAs. Results were normalized to U6. Values are expressed as fold induction of miRNAs over untreated controls using the ΔΔCq method and data shown is an average of at least 3 experiments.

	Kcal/Mol	
Site	miRanda	RNAHybrid
miR-29a 10009-10056	-22.07	-
miR-29a 10058-10090	-24.08	-20.5
miR-29b 10058-10090	-21.32	-20.3
miR-9 9744-9770	-22.6	-21.9
miR-146a 9678-9711	-29.47	-26.7
miR-146a 9985-10025	-23.89	-

Table S1. Binding energies for each miRNA site within the SIV LTR sequence, predicted by miRanda and RNAhybrid. The minimum binding energy threshold was set to -20 kcal/mol.

V. SUMMARY AND FUTURE DIRECTIONS

The work included in this dissertation demonstrates how miRNAs, discovered only a short time ago, have the ability to target and regulate the expression of not only endogenous host genes, but also that of non-evolutionary targets, such as viruses. We show here that miRNA expression can differ dramatically between cells that have the ability to become productively infected with HIV/SIV and those that do not. Our work also identified functional miRNA binding sites within the SIV genome, and addition of these miRNAs to SIV-infected cells leads to a decrease in virus production. We found miRNA regulation of IFNβ, a major type I IFN, and regulation by IFNβ and TNFα, for some of these same miRNAs. This work underscores the multifaceted and complex nature of the immune system and the importance of understanding the fine-tuning mechanisms underlying its action.

Results here show miRNA regulation of SIV infection. Further study could be done to determine if this effect is in fact due to direct miRNA targeting of SIV mRNAs or of another factor necessary for virus replication. Mutations predicted to disrupt miRNA targeting of seed binding regions could be made to the SIV genome. If SIV RNAs contain true functional miRNA binding sites, then individual miRNAs should have no impact on replication of the virus containing the mutation for that miRNA's specific seed-binding site. These studies may be complicated because the binding sites for all four miRNAs lie near or within the trans-activation response (TAR) element. Because this stretch of viral sequence is essential for productive infection, it may be difficult to introduce mutations that impact miRNA binding without negatively impacting virus replication.

It would also be interesting to investigate whether miRNA inhibition of SIV replication is more beneficial to the host or to the virus. Infected CD4+ T cells, and potentially macrophages, can each serve as a latent reservoir for HIV and SIV. Latency allows viral DNA to persist in host cells with the ability to have a rebound in virus replication upon activation of the resting/latent cell. The miRNA action we observed may contribute to a latent state, thereby ensuring survival of the viral genome. Searching for viral escape mutants by deep sequencing of the viral genome, after miRNA treatment of infected cells, would be one way to analyze this. The presence of virus escape mutants would suggest that the miRNA action is detrimental to the virus life cycle.

The impact of miRNAs on viral pathogenesis is a new area of research. The virus-host relationship is an elaborate web of interactions and miRNAs are proving to be key players. The immune response requires a delicate balance of eliminating infection while limiting damage due to inflammation and cell death. Understanding the nuance of the body's response to infection will lead to more effective treatments in the future.

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Publications

1. Jonathan Liu, **Jeanne M. Sisk**, Lucio Gama, Janice E. Clements and Kenneth W. Witwer, 2013, "Tristetraprolin expression and microRNA-mediated regulation during simian immunodeficiency virus infection of the central nervous system", *Molecular Brain*, 6:40.
2. **Sisk JM,** Witwer KW, Tarwater PM, Clements JE, 2013, "SIV replication is directly downregulated by four antiviral miRNAs", *Retrovirology*, 10(1):95.

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3. **Sisk JM**, Clements JE, Witwer KW, 2012, "MiRNA profiles of monocyte lineage cells are consistent with complicated roles in HIV-1 restriction", *Viruses*, 4: 1844-1864. PMCID: PMC3497032
4. Witwer KW, **Sisk JM**, Gama L, Clements JE, 2010, "MicroRNA regulation of IFN-beta protein expression: rapid and sensitive modulation of the innate immune response", *J Immunol*. Mar 1;184(5):2369-76. PMCID: PMC3076721
5. Malloy PJ, Wang J, Peng L, Nayak S, **Sisk JM**, Thompson CC, Feldman D, 2007, "A unique insertion/duplication in the VDR gene that truncates the VDR causing hereditary 1,25-dihydroxyvitamin D-resistant rickets without alopecia", *Arch Biochem Biophys*. Apr 15;460(2):285-92. PMCID: PMC1892178
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Conference Abstracts

1. Kenneth W. Witwer, Erin Buchanan, Diego Espinosa, **Jeanne M. Sisk**, Adam Moyer and Janice E. Clements, "Expression and Regulation of Host Restriction Factor SAMHD1 During Lentiviral Infection of Astrocytes and Macrophages", March 2013, *Conference on Retroviruses and Opportunistic Infections (CROI)* – poster author
2. **Jeanne M. Sisk**, Kenneth W. Witwer and Janice E. Clements, March 2012,

"IFNβ Induces Anti-SIV microRNAs as Part of the Innate Immune Response", *Conference on Retroviruses and Opportunistic Infections (CROI)* – poster presentation

3. **JM Sisk**, KW Witwer, JE Clements, December 2011, "microRNAs and the Innate Immune Response to SIV Infection", *HIV Persistence During Therapy* – oral presentation
4. **Jeanne M. Sisk**, Kenneth W. Witwer and Janice E. Clements, February 2011, "microRNAs and the Innate Immune Response to SIV Infection in the Brain", *Keystone – microRNAs and Human Disease* – poster presentation

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